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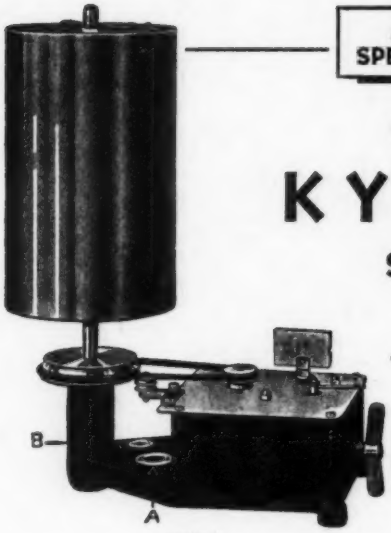

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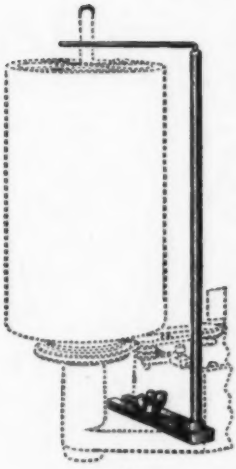

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BIOLOGICAL BULLETIN

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EFFECT OF ADENOSINETRIPHOSPHATE (ATP) ON THE ENDOGENOUS OXYGEN UPTAKE OF DEVELOPING GRASSHOPPER EMBRYOS[1]

JOSEPH HALL BODINE AND WILLIAM LIONEL WEST

Zoological Laboratory, State University of Iowa, Iowa City, Iowa

A phosphate transfer system has been found in practically every tissue thus far investigated (Lardy, 1949). It functions directly or indirectly in almost every phase of metabolism and has become so well established that its presence and functions are very often inferred without further demonstration. Previous studies in this laboratory have revealed that homogenates of the embryos of the grasshopper, *Melanoplus differentialis,* in 0.25 *M* sucrose can oxidize hexose phosphates to a greater extent than glucose (Bodine and West, 1953). Bodine and Thompson (1938) reported that labile phosphate is found in both the embryo and yolk while Lu and Bodine (1953) found a gross transformation of phosphorus from yolk to embryo. However, very few direct observations seem to have been made on the chemical or functional nature of the phosphate transfer system in developing organisms. Recently Albaum and Kletzkin (1948) and Calaby (1951) confirmed the presence of ATP in insects. Humphrey and Siggins (1949) presented indirect evidence that glycolysis in insect muscle involves the phosphate transfer system while Sacktor (1953) describes a specific ATPase in flight muscle mitochondria.

The present paper is concerned with results of a study on the effects of ATP on the endogenous O_2 uptake of grasshopper embryos at different developmental stages. These results are discussed in the light of a functional phosphate transfer system as exhibited by other organisms.

Materials and Methods

Embryos of the grasshopper, *Melanoplus differentialis,* were dissected from eggs in Ringer solution (buffered at pH 6.8 with *M*/15 phosphate) and washed free of adhering yolk (Bodine and Boell, 1934, 1936). The washed embryos, suspended in a suitable volume of the selected medium, were homogenized using a Pyrex glass tube with a tight fitting selenite rod as a pestle. The pestle rotated at 1150 r.p.m. and the time of homogenation was two and one-half minutes at 0° C.

Two suspension media were used, Ringer solution containing 0.0035 *M* mag-

[1] Aided by a grant from the National Institutes of Health.

nesium chloride (pH 6.8) and 0.25 *M* sucrose containing 0.0035 *M* magnesium and 0.0035 *M* calcium chlorides and 0.03 *M* phosphate (pH 6.8) (Bodine and West, 1953). One hundred intact embryos or homogenates containing the equivalent of one hundred embryos per cubic centimeter were used throughout this investigation.

Oxygen uptake determinations (air as gas phase) were carried out by standard Warburg techniques at 25° C.; 0.5 cc. of substrate were tipped from the sidearm to make the final volume of the reactants 1.5 cc.

Adenosinetriphosphate (ATP) (sodium salt) was obtained from the Sigma Chemical Company, St. Louis, Missouri.

Results

The effect of ATP on the endogenous O_2 uptake of intact embryos (mitotically active or blocked) was investigated over a range of concentrations from 2.5 to 10.0 μmoles per 1.5 cc. Data for a typical experiment are summarized in Table I. From an examination of this table it is apparent that ATP has little, if any, significant stimulating effect on the respiration of intact embryos either in 0.25 *M* sucrose or Ringer solution. This lack of effect may be related to or conditioned by the permeability of the intact embryo to these reagents.

Table I

	Prediapause (17 days)		Diapause (40 days)		Postdiapause (3 days)	
	E	H	E	H	E	H
Control (sucrose)	17.0	5.7	10.0	4.6	17.4	5.9
ATP	16.0	9.4	9.3	6.5	16.5	13.3
ATP+glu	15.0	10.2	9.1	8.0	16.7	14.3
ATP+glu−1−PO_4	19.6	13.2	14.2	9.7	18.2	13.6
Glucose−1−PO_4	18.8	8.5	15.2	6.5	18.5	9.0

Shows O_2 uptake (cc.) for 100 minutes for prediapause, diapause and postdiapause embryos (E) and their homogenates (H) in 0.25 *M* sucrose plus Mg^{++} and Ca^{++} after addition of ATP, 5 μmoles per flask; glucose 1.0%; glucose-1-phosphate 0.5%. Stimulation due to hexosephosphate has previously been pointed out (Bodine and West, 1953). Data in table are taken from one series of experiments and represent averages from a minimum of 8 determinations. All data from different experiments have been statistically analyzed and differences, indicated in text, found to be significant.

The effect of ATP on the endogenous O_2 uptake of homogenates of embryos in 0.25 *M* sucrose is strikingly different from that of the intact embryo. ATP augmented the endogenous respiration of homogenates in sucrose (Table I). The concentration effect was found to be quite variable at high concentrations and this is attributed to the formation of clumps which entangled the mitochondrial elements, thus preventing or interfering with electron transfers. This clumping effect was more apparent in diapause and postdiapause stages at the 10 μmole level of ATP. Clumping is believed to be caused by an involvement of embryonic actin, myosin, and ATP and is given support by the observation that clumping seldom occurred in the prediapause stages before 17 days at which time the percentage

stimulations were more consistent. Maximal augmentation of respiration was obtained at the 5 μmole level where the clumping effects were absent. This concentration has been selected as optimal in these experiments. ATP, when tipped from the sidearm, produces a lag before maximum augmentation of oxygen uptake. Homogenates made in 0.25 *M* sucrose plus ATP (employed only for diapause) showed a greater oxygen uptake than when ATP was added to the sucrose homogenate. The magnesium ion was necessary for maximal stimulation by ATP.

Homogenates made in Ringer and Mg^{++} showed no stimulation of endogenous oxygen uptake when ATP was added.

Combinations of ATP and glucose produced no marked hexokinase activity in either the intact embryo or its homogenate. Similarly, no marked phosphoglucokinase activity was apparent.

Washed nuclei in sucrose or Ringer showed no response in their endogenous oxygen uptake to these concentrations of ATP.

Discussion

The exact nature of the labile phosphorus compounds of the phosphate transfer system in this material has not yet been satisfactorily demonstrated, due largely to various inherent technical difficulties. However, it is known that the labile phosphorus component of the embryo is adsorbed on activated charcoal (method of Crane and Lipmann, 1953), which is a characteristic of the adenosine-containing nucleotides (unpublished data). The ability of hexosephosphates to stimulate endogenous respiration of intact embryos is quite unusual and no active mechanism has been revealed (Bodine and West, 1953). ATP, unlike the hexosephosphates, seems to have no stimulating effect on the endogenous respiration of the intact embryo (mitotically active or blocked). Similarly, glucose plus ATP gave no increased endogenous O_2 uptake, indicating no marked hexokinase activity at or near the cell membrane.

ATP markedly stimulates endogenous respiration of the homogenates in 0.25 *M* sucrose (Mg^{++}, Ca^{++}) and thus one can infer a functional phosphate transfer mechanism. This effect may take place through "active" phosphorylation of endogenous substrates, making them more available for oxidation, or "active" dephosphorylation by a specific ATPase, increasing the concentration of high energy phosphate acceptors (ADP + AMP) and permitting the oxidation of available endogenous substrates or a combination of both. (This discussion presupposes that oxidation and phosphorylation are linked.) Studies are in progress to clarify this point.

ATP does not stimulate the endogenous respiration of Ringer homogenates. The mitochondria lose their morphological integrity in this medium and show a marked functional difference to added succinate and hexosephosphates. Thus structural integrity of the mitochondria in this material seems related to their functions.

Combinations of glucose or hexosephosphates with ATP in sucrose homogenates yield variable results. No effort was made to remove the endogenous substrate, and at present it can be said that there appears to be no marked hexokinase or phosphohexokinase activity in this material.

SUMMARY

1. A study has been made on the effects of ATP on the endogenous O_2 uptake of grasshopper embryos and homogenates at different developmental stages.
2. ATP has little, if any, effect upon the O_2 uptake of the intact embryo.
3. ATP augments the O_2 uptake of homogenates in sucrose.
4. The magnesium ion is necessary for maximal stimulation of ATP.

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NUTRITIONAL STUDIES ON THE AMOEBO-FLAGELLATE, TETRAMITUS ROSTRATUS [1]

MORGAN M. BRENT [2]

Department of Zoology, University of California, Berkeley 4, California

Nutritional studies on amoebae have lagged far behind those on ciliates and flagellates. The earliest controlled work on the nutrition of members of the Sarcodina concerned the cultivation of single species of amoebae in the presence of bacterial mixtures. Here, most emphasis was placed upon the culture medium itself, the living bacteria being considered as obligate factors for growth of the phagotrophic forms. Scattered reports of axenic (Dougherty, 1953) cultures of amoebae have been recorded in the literature, but the earliest accounts have not been corroborated by other investigators. Included in these is the reported cultivation of some amoebae *in vitro* on sterile mammalian tissues by Williams (1911). She believed that these amoebae were parasites, but this is doubtful in the light of present research upon known parasitic forms. Oehler (1924) claims to have grown several unknown species of free-living amoebae under axenic conditions upon water-agar containing coagulated serum. As far as is known this work has not been re-investigated.

One of the first species to be grown free of living bacteria and which can be obtained today is the free-living amoeba, *Acanthamoeba castellanii*. Castellani (1930) found that this amoeba was capable of growing upon a killed bacterium and dead yeast. Cailleau (1933b) succeeded in obtaining an entirely liquid medium for *A. castellanii* consisting of peptone and added salts. This has been modified somewhat by Storm, Hunter and Cowperthwaite (1951) who grew the amoeba upon a medium containing acid-hydrolysate of gelatin and a low concentration of skim milk. The same authors (1951) have also reported growth of *Hartmannella rhysodes* upon an autoclavable liquid medium containing hydrolysates of gelatin and free oleate esters.

Reich (1935) reported axenic cultivation of the soil amoeba, *Mayorella palestinensis*, upon a clear medium consisting of peptone, a balanced salt solution, and added dextrose. The latter component appeared indispensable for good growth.

On the other hand, many investigators have failed to grow amoebae axenically. Among these is Wherry (1913) who, in preliminary experiments, could not succeed in growing an amoebo-flagellate (probably *Naegleria gruberi*) free of the bacterial flora. Rice (1935) succeeded in growing the marine amoeba, *Flabellula mira*, in monobacterial cultures, but failed to cultivate this form axenically by adding either killed bacteria or various amino acids to the basal medium.

[1] A portion of a dissertation presented to the graduate school of Northwestern University in partial fulfillment of the requirements for the Ph.D. degree in biology. The author wishes to express his appreciation to Dr. William Balamuth for his helpful suggestions and interest during the course of this investigation.

[2] Present address: Department of Zoology, University of California, Berkeley, California.

Although a few of the parasitic flagellates have been cultured axenically *in vitro*, the nutrition of the parasitic amoebae has provided a more difficult problem. In the case of *Entamoeba histolytica*, the amoebae apparently require not only certain metabolites provided by the basal medium but also an anaerobic environment mediated by the associated bacteria (Snyder and Meleney, 1943), as well as the bacterial bodies themselves (Shaffer, 1952). As far as is known at present, *E. histolytica* has not been grown *in vitro* free of the influence of living protoplasm (other Protozoa, bacteria, embryonic tissue cultures).

As one approach to the nutrition of the parasitic amoebae it seemed desirable to investigate a free-living coprophilic form, since these Protozoa pass sporadically through the lower digestive tract of various animals. Although sharing the same environment as intestinal parasites in this respect, they seem to be incidental guests of their hosts and might conceivably stand at the threshold of parasitism. Little work has been done on the amoebo-flagellate, *Tetramitus rostratus*, apart from morphological and cytological studies. According to most investigators this organism is a coprophile which actively feeds and multiplies in its trophic stages upon substrates rich in organic material. It has also been found in fecal cultures of various animals by Bunting (1922) and Rafalko (1951). In view of these facts this organism provided an excellent opportunity for a nutritional investigation which might link free-living and parasitic forms.

Materials and Methods

The strain of *Tetramitus* used in this investigation was obtained in December, 1950 by Dr. William Balamuth from Miss Lois Norman at the Communicable Disease Center in Chamblee, Georgia. It had been found in a "sterile" urine specimen in Austin, Texas in the same year. Routine cultures of the wild stock were maintained at room temperature upon a medium consisting of 0.5% Difco yeast extract plus 0.5% Difco peptone (YP) made up in distilled water. The diphasic nature of this organism was first demonstrated by Bunting (1922, 1926), who pointed out a reversible amoeba-to-flagellate transformation in its life cycle. Although both stages of the present strain appeared in the bacterized maintenance broth only the amoeboid form was present on solid agar-containing media or under axenic conditions.

Handling of cultures

In order to obtain large numbers of bacteria-free amoebae for use in nutritional studies it was thought advisable to establish the trophozoites in a two-membered culture with a penicillin-sensitive bacterium. Washed cysts from the wild stock of *Tetramitus* were first sterilized by a series of chemical procedures. These included successive treatment at 23° C. with 1 : 50,000 $HgCl_2$ for one hour and with 1 : 5000 $KMnO_4$ for 30 minutes. The cysts were then implanted into a tube of YP broth containing *Micrococcus pyogenes* var. *aureus*. Bacteria-free amoebae could be obtained from this culture by inoculating 6- to 14-day-old trophozoites onto plates of YP medium containing 1.5% agar plus 500 units/ml. of penicillin and incubating them for 3 days at room temperature. Their sterility was routinely tested by

inoculating them into Difco fluid thioglycollate medium and Difco stock culture agar (SCA).

In assaying prepared media the general plan was as follows: Penicillin-treated amoebae were gently flushed off the agar surface with sterile tap water and pooled in a test tube. The organisms were inoculated in 0.1-ml. amounts into 150 × 18 mm. cotton-stoppered tubes containing 5 ml. of sterile test broth. The viability of the treated amoebae used in each experiment was tested by inoculating them into broth containing living bacteria. All cultures were incubated in a moist chamber at 30° C. in a slanting position and observed at regular 2- to 4-day intervals for as long as 10 to 14 days. Transplants were made in approximately 0.2-ml. amounts. In order to eliminate carry-overs consideration was given only to the fourth subculture when evaluating positive results. Positive cultures were always re-checked for bacterial sterility in fluid thioglycollate medium and SCA.

As required in population runs, amoebae from broth cultures were counted upon four hemocytometer fields and the results averaged.

Preparation of media

In axenic assays, initial experiments were designed to modify the concentration of components of the original YP medium. In some cases substitution for the yeast extract was made with Anheuser-Busch autolyzed or pepsin-digested yeast. Protein digests including BBL trypticase, BBL phytone, Difco proteose-peptone and Difco tryptone in concentrations from 0.1% to 3.0% were substituted for the peptone fraction. More complex media involved the addition of Cerophyl (dehydrated cereal grass leaves), liver extract, cream, whole egg, blood and selected vitamins to the basic YP medium in varying concentrations.

A review of the literature suggested types of media which have been used to grow Protozoa axenically. Pressed-yeast juice was prepared according to the method of Johnson and Baker (1942), in which they cultivated *Paramecium multimicronucleatum*. Variations of their medium, sterilized by Selas filtration, consisted of adding the concentrated juice to distilled water in amounts ranging from 3.0% to 50.0% in approximately two-fold concentrations. Cailleau (1933a, 1933b) reported growth of *A. castellanii* upon two kinds of media. Her first medium (1933a) and variations of her later medium (1933b) were tried. Trypticase, phytone and peptone in concentrations from 1.0% to 3.0% were substituted for the peptone fraction in her newer medium. Reich's medium (1935) for *M. palestinensis* was also utilized in the screening procedures.

All media except Johnson's and Baker's pressed-yeast juice were sterilized by autoclaving for 20 minutes at 15 pounds pressure. The hydrogen-ion concentrations were not critically controlled but were adjusted when possible to approximate neutrality.

Various species of dead bacteria were utilized as a food source in many of the experiments. The organisms were grown upon YP agar in large petri dishes for 24 to 48 hours. After maximum growth was obtained the cells were scraped off the plates, suspended in tubes of distilled water and autoclaved for 30 minutes at 15 pounds pressure.

Preparation of bacterial hydrolysates and extracts

It later became evident that certain substances present in the dead bacterial cells (particularly in *Bacillus cereus* and *B. subtilis*) were required for growth of the amoebae. The *B. cereus* cells were fractionated in the following manner: Thirty grams of freshly harvested bacteria were added to an equal weight of alumina and the aggregate ground by hand with dry ice. One hundred and sixty milliliters of distilled water were added to the crushed cells and the alumina centrifuged out. The suspended cells were divided among three beakers in equal amounts and enough 1 N HCl and 1 N NaOH added separately to two of the containers to make 0.1 N solutions of acid and base, respectively. The contents of the third beaker were adjusted to pH 7.00. All three of the vessels were autoclaved for 30 minutes at 15 pounds pressure yielding acid-, alkaline-, and neutral-hydrolyzed fractions. The contents from each beaker were then divided into two parts:

(a) One-half of the cell suspension from each beaker was subjected to dialysis through a Visking casing for 28 hours at 4° C. in liter beakers with four changes of cold distilled water. The dialysates were discarded for the purposes of these experiments. After dialysis the pH of the three non-dialyzable [3] fractions containing the cell-residues was adjusted to neutrality. The cell-residues were collected and washed and the non-dialyzable supernatants concentrated to 5 ml. by boiling.

(b) The pH of the remaining non-dialyzed acid, alkaline and neutral fractions was also adjusted to neutrality; the cell-residues were collected and the non-dialyzed supernatants concentrated in a similar manner.

All fractions were then re-autoclaved and stored at 4° C.

In later investigations it became necessary to extract the fat-soluble fractions of the neutral-hydrolyzed non-dialyzed supernatants with various fat solvents. This was accomplished by shaking each supernatant with an equal volume of solvent, collecting the latter and repeating the procedure several times. The solvent was evaporated to dryness and the sediment brought up to the original volume with distilled water. Before extracting with alcohol or acetone it was first necessary to evaporate the supernatants to dryness; the insoluble materials were then centrifuged out.

Vitamins, purines, pyrimidines and amino acids

In experiments requiring special nutrients, mixtures of vitamins, purines, pyrimidines and amino acids were added in place of certain bacterial fractions. A stock solution of vitamins was prepared consisting of 50 mg. each of choline·HCl, folic acid, inositol, nicotinic acid, paramino-benzoic acid, calcium pantothenate, pyridoxine·HCl, riboflavine, thiamine·HCl and 25 μg of biotin. The purine-pyrimidine mixture consisted of 50 mg. each of adenine sulfate, cytidylic acid, guanine, thymine, uracil and xanthine. Both of the above mixtures were separately suspended in 500 ml. of distilled water, filtered through No. 03 Selas filters and stored at 4° C. The amino acid stock mixture contained 50 mg. each of L-asparagine, DL-alanine, L-cystine, L-glutamic acid, DL-methionine and DL-tryptophane. The above acids were added to 50 ml. of distilled water and autoclaved for 10 minutes at 15 pounds pressure.

[3] *Non-dialyzable* refers to a retained fraction after being subjected to dialysis, as opposed to *non-dialyzed* fractions which were not subjected to dialysis.

Experiments and Results

Selection of a medium

Experiments with most empirical formulae and those media already known to support axenic growth of the free-living Protozoa described above, proved unsuccessful for *Tetramitus*. Axenic growth of *Tetramitus* could be obtained, however, when washed autoclaved *Bacillus cereus* was added to a medium consisting of 0.5% yeast extract, 0.5% peptone and 1.0% Wilson liver concentrate (N.F.). In the yeast-peptone-liver medium (designated as YPL) sterile young amoebae were 2–3 times larger than normal (60 μ in diameter) and usually appeared opaque and immobile, but they became progressively more active as the cultures became older. Through varying the pH from 5.5 to 7.9 it was found that better growth could be ob-

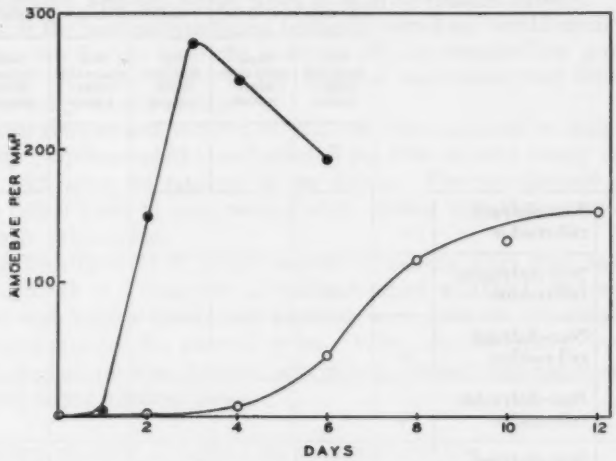

Figure 1. Comparison of growth of populations of *T. rostratus* with living vs. autoclaved *B. cereus* in 5 ml. of YPL medium at 30° C. Inoculum-= 870/ml. ●: Growth with living bacteria; o: Growth with dead bacteria. Note: Each point represents a separate tube.

tained near neutrality (6.8–7.3). Altering the temperature from 20° C. to 35° C. demonstrated that optimum growth (150 per mm.^{3}) could be reached in approximately 12 days at 30° C.

A distinct lag phase was observed when the amoebae were grown with dead *B. cereus* (Fig. 1), which was considerably longer than that obtained with living bacteria. This would seem to indicate that although this medium is capable of supporting growth and reproduction of the amoebae, it is far from perfect and is incapable of supplying all the factors derived from cultures containing living bacteria. Preliminary experiments to shorten this lag with Selas-filtered, preconditioned bacterial cultures have not proved promising.

Attempts were made to substitute other autoclaved bacteria for *B. cereus* in YPL broth. Considerable growth of the amoebae could be obtained with killed *Escherichia coli, Neisseria catarrhalis, Sarcina lutea* and *Bacillus subtilis* by culturing

at 8-day intervals. No growth could be obtained with autoclaved *Pseudomonas fluorescens*. Substitution of autoclaved yeast Harris, Anheuser-Busch and Difco whole yeast failed to replace this bacterial factor.

Although the liver portion of the medium could be reduced to 0.25% without appreciably affecting growth, its complete elimination resulted in poor numbers of amoebae. However, amoebae have been cultured in this medium (YP) for two months with the addition of large amounts of autoclaved *B. cereus* or *B. subtilis* at each sub-culture. The liver-deficient medium would not support growth of the

TABLE I

Growth-supporting capacity of fractions of Bacillus cereus for Tetramitus rostratus in sterile yeast-peptone-liver medium

			Neutral hydrolysis		Acid hydrolysis		Alkaline hydrolysis	
			Non-dialyzed supernatant	Non-dialyzable supernatant	Non-dialyzed supernatant	Non-dialyzable supernatant	Non-dialyzed supernatant	Non-dialyzable supernatant
			−	−	−	−	−	−
Neutral hydrolysis	Non-dialyzed cell-residue	* −						
	Non-dialyzable cell-residue	−	+	−	−		−	
Acid hydrolysis	Non-dialyzed cell-residue	+						
	Non-dialyzable cell-residue	+			+			
Alkaline hydrolysis	Non-dialyzed cell-residue	* −					−	
	Non-dialyzable cell-residue	−						−

* Present only in initial culture.
\+ Growth of amoebae.
− No growth of amoebae.
Note: All supernatants were added as 1 pt. to 4 pts. of YPL medium. Cells were added in excess (approx. 0.1 ml. wet mass).

amoebae with added autoclaved *E. coli* or *N. catarrhalis*. Apparently *B. cereus* and *B. subtilis* contain at least one or more factors found in liver extract which seem to be lacking in the two non-spore-forming bacteria. These factors remain to be elucidated.

Bacterial hydrolysates

Since something in the bacterial cell appeared necessary for sustained growth of *Tetramitus* in YPL medium, partial hydrolysis of *B. cereus* was carried out as

described, in order to separate possible essential fractions. It was found at the outset that the substance(s) survived autoclaving for as long as 50 minutes, and a thorough washing of the intact cells failed to remove any activity.

It can be seen from Table I that amoebic growth resulted in YPL medium with the addition of the neutral-hydrolyzed non-dialyzable cell-residue to the neutral-hydrolyzed non-dialyzed supernatant. Although the non-dialyzed supernatant was routinely added as one part to four parts of YPL medium (by choice), it was found that it would maintain trophic growth in one-half this amount as long as the cell-residue was added. No growth could be obtained using the supernatant alone even when raised to 50% of the total medium. It can also be seen that the non-dialyzable supernatant is completely inactive whether used alone or with the non-dialyzable cell-residue. This residue still retained a small quantity of fat which was demonstrated with the Sudan Black B stain of Burdon (1946). The results obtained with the neutral-hydrolyzed fractions, therefore, would seem to indicate that at least two factors from the bacterial cell are required for growth of the amoebae: one dialyzable found in the bacterial supernatant and the other non-dialyzable found in the cell-residue.

Macerated cells treated with 0.1 N HCl and then subjected to dialysis retained their activity. Microscopical examination of the cells revealed mostly disintegrated cell husks with some fat retained in the debris. The non-dialyzed supernatant from these cells (Table I) was inactive when utilized with the neutral-hydrolyzed non-dialyzable cell-residue.

Alkaline hydrolysis of *B. cereus* seemed to destroy more than one factor required for growth of *Tetramitus*. Combinations of alkaline-hydrolyzed fractions together or with neutral-hydrolyzed fractions were inactive. Examination of the cell fragments revealed the absence of fat. When alkaline-treated fractions were added with normal autoclaved whole cells growth ensued, demonstrating that there is no toxicity factor involved here.

1. Preliminary attempts to replace the dialyzable fraction

Utilizing the lead obtained from neutral hydrolysis, experiments were performed to discover the nature of the factor(s) which were removed by dialysis. In these experiments the non-dialyzable fraction was provided by the non-dialyzable cell-residue. The possible dialyzable factors were sought separately in a mixture of vitamins, a mixture of purines and pyrimidines and amino acids. The vitamin mixture described above added in 0.002-ml., 0.02-ml. and 0.2-ml. amounts per 5 ml. of YPL medium gave no indication of supporting growth. The mixture of purines and pyrimidines added in 0.02-ml., 0.2-ml. and 2.0-ml. amounts to the vitamin-containing medium also failed to maintain growth. Mixed amino acids added in 0.25-ml., 0.5-ml. and 1.0-ml. amounts in combination with the vitamins and the purine-pyrimidine mixture gave no promising results.

In view of the fact that the substituted mixtures showed no activity, it was postulated that non-dialyzable fractions in the bacterial supernatant were also required for growth. This immediately suggested lipoidal material. To test this possibility, the non-dialyzable supernatant was utilized with the non-dialyzable cell residue as non-dialyzable fractions. No growth of the amoebae resulted with the addition of the vitamin mixture to these fractions in YPL medium. This was

re-investigated with the lipoid-extracted portions of the supernatant added as one part to four parts of YPL medium. In no case was growth of *Tetramitus* observed when either benzene-, alcohol-, ether-, or acetone-extracted supernatant was added to the vitaminized medium containing the non-dialyzable cell-residue.

2. Preliminary attempts to replace the non-dialyzable fraction

In routine investigation of the non-dialyzable fraction, neutral-hydrolyzed non-dialyzed supernatant was added to every tube of YPL broth as one part to four parts of medium; and in addition the vitamin mixture was added in a concentration of 0.1 ml. per 5 ml. of broth.

In order to eliminate the possibility that an essential metal might be tied up in the bacterial residue, the cells were completely ashed by flaming them in a Pyrex tube and then added to the culture medium. No growth of *Tetramitus* could be obtained. The separate addition in 1.0% and 5.0% proportions of peptone, tryptone, tryptose, proteose-peptone, yeast extract, trypticase and phytone failed to replace this factor. Skim milk in concentrations of 0.01% to 0.5% has also failed. Experiments are planned to replace this factor with known proteins and polysaccharides.

Discussion

Although only preliminary experiments have been carried out on the nutritional requirements of *Tetramitus*, it has been shown that the dead bacterial cell provides some essential constituent(s) for axenic growth of the amoebae in the yeast-peptone-liver medium. The types of killed bacteria it can use in this medium are non-specific since it has utilized gram-negative and gram-positive representatives of the cocci group, gram-positive spore-formers and a gram-negative coliform. It appears, however, that although *Tetramitus* will grow in YPL medium with most of the species of autoclaved bacteria investigated, only the spore-formers were utilized by the amoebae in the liver-deficient medium. Since *Bacillus cereus* and *B. subtilis* contained large quantities of fat, it is interesting to speculate whether they provide certain lipoidal substances present in the liver extract. This hypothesis should be tested by adding to the YP medium the fat-extracted portions of these cells together with the autoclaved non-spore-forming bacteria.

The finding that *Tetramitus* can utilize certain heat-stable metabolites found in microorganisms is not a new one when considering Protozoa in general. Johnson (1936) was able to obtain growth of the holotrichous ciliate, *Glaucoma ficaria*, in suspensions of 11 species of dead bacteria as well as 6 species of dead flagellates, using a balanced salt solution as a basal medium. Glaser and Coria (1935) established *Paramecium caudatum* and *P. multimicronucleatum* as well as other Protozoa upon a medium containing dead yeast cells as an indispensable nutrient. Van Wagtendonk and Hackett (1949) secured axenic growth of *Paramecium aurelia* but had to provide a 24-hour-preconditioned, autoclaved lettuce infusion culture of *A. aerogenes* with autolyzed yeast. When either component was omitted growth of the ciliates stopped, indicating essential substances other than the bacterial fraction.

It is evident from the preliminary work on *Tetramitus* that this organism is not

as fastidious in its growth requirements as the parasitic amoebae. The fact that it can grow and multiply aerobically in the absence of other living protoplasm tends to make the nutritional approach an easier one. With *Entamoeba histolytica*, on the other hand, nutritional studies have been hampered by its dependency upon associated living organisms and upon its extreme sensitivity to oxygen. Recently a certain substance(s) in dead bacteria has also proved essential for this latter species. Karlsson, James and Anderson (1952) have shown that when an autoclaved culture of a streptobacillus in liver-proteose-peptone medium was used as a substrate for *E. histolytica* under antibiotic treatment to suppress bacterial growth, fair growth of the amoebae resulted. Use of filtered media resulted in the loss of activity, suggesting the active material was present in the cells. Later Karlsson (1952) showed that 90% of the cells' activity was destroyed during the first 20 minutes of autoclaving. In the present work on *Tetramitus* the bacterial fractions are clearly heat-stable, since prolonged autoclaving for 50 minutes does not seem to destroy their activity. The streptobacillus factor for *Entamoeba* was completely destroyed by 0.1 N NaOH in 5 minutes at room temperature but could withstand treatment with 0.1 N HCl for one hour, suggesting similarity to those factors found in the *B. cereus* cell. It is also interesting to note that Karlsson's streptobacillus fraction could not be extracted with fat solvents.

Although no definite decision can as yet be made, the dialyzable factor in the cell-extracted supernatant of *B. cereus* suggests some protein fragment, for example, a polypeptide or some other dialyzable substance of relatively low molecular weight. There exists the possibility that several essential substances, both dialyzable and non-dialyzable, may be present in the supernatant. Collection and analysis of the dialysates would be of value in elucidating these fractions. The supernatant fraction appears to remain with the cell-residue when subjected to acid hydrolysis (Table I). Further evidence for this was shown by complete inactivity of the acid-hydrolyzed non-dialyzed supernatant. Preliminary experiments have shown that this acid fraction contained no inhibiting substance when added to acid-hydrolyzed cells.

The non-dialyzable fraction in the cell residue would suggest substances either proteinaceous or polysaccharide in nature. More complete analysis of this fraction is required before arriving at any conclusions.

Summary

1. *Tetramitus* was cultivated indefinitely under axenic conditions upon a medium consisting of 0.5% Difco yeast extract, 0.5% Difco peptone and 1.0% Wilson liver concentrate (N.F.) with selected types of autoclaved bacteria.

2. The liver concentrate could be eliminated with subsequent reduction in trophic growth, only if killed *B. cereus* or *B. subtilis* was utilized as the bacterial fraction. Other bacteria (*Neisseria catarrhalis, Escherichia coli*) could not be substituted for these spore-formers in this medium.

3. It was found that the *B. cereus* cell contained at least two heat-stable fractions necessary for growth; neutral hydrolysis yielded a non-dialyzable fraction found in the cell-residue and a dialyzable fraction found in the bacterial cell supernatant.

4. Both factors were stable to autoclaving with 0.1 N HCl for 30 minutes at

15 pounds pressure and were retained in the acid-treated cell-residue. Alkaline hydrolysis destroyed activity of all the fractions.

5. Preliminary attempts have failed to substitute for the factors found in the neutral-hydrolyzed bacterial supernatant by employing selected vitamins, amino acids, purines and pyrimidines.

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THE PENETRATION OF RADIOACTIVE PHOSPHATE INTO MARINE EGGS [1, 2]

SUMNER C. BROOKS AND EDWARD L. CHAMBERS [3]

Department of Zoology, University of California, Berkeley, California

Needham and Needham (1930) showed that the gastrulae and plutei of the echinoderm *Dendraster excentricus* have a higher total phosphate content than that of the unfertilized eggs. These authors suggested that the increased phosphate content of the larvae was related to the formation of the skeletal spicules.

Brooks (1943a) obtained results, using radioactive phosphate, which indicated that soon after first cleavage in the fertilized eggs of *Arbacia punctulata,* the intake of radiophosphate was accelerated. During the winter of '46 to '47 these experiments were repeated using more refined methods and the eggs of several different species of sea urchins, as well as the eggs of the gephyrean worm, *Urechis caupo.* The results are presented in this paper. Radiophosphate was found to enter the fertilized eggs of sea urchins more than one hundred times faster than it entered the unfertilized eggs (Brooks and Chambers, 1948). There was no evidence for alternating phases of intake and loss of phosphate ions, such as have been reported to occur during the early period of ion uptake by single *Nitella* cells and by uniform populations of egg cells (Brooks, 1939a, 1939b, 1940, 1943a, 1943b). The previously obtained results are to be ascribed to the considerable variability inherent in the methods which had been used (Brooks, 1951).

Independently Abelson (1947), using the eggs of *Arbacia punctulata,* and Lindberg (1948), using the eggs of *Psammechinus miliaris,* demonstrated the relatively more rapid penetration of radioactive phosphate into the fertilized, as compared to the unfertilized, sea urchin egg.

Methods

Materials. Eggs shed from the ovaries of the freshly collected Pacific coast sea urchins *Strongylocentrotus purpuratus, S. franciscanus* and *Lytechinus pictus,* and eggs obtained from the "egg collectors" (MacGinitie, 1935) of the worm *Urechis caupo* were used. The eggs were strained through cheese cloth and washed four times by centrifugation. In each experiment eggs from only a single animal were used, unless otherwise stated.

Conduct of the experiment. Egg suspensions containing 1 ml. eggs/liter sea water were used. Impaired development occurs if the concentration exceeds 5–6

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[3] Work done while Porter Fellow of the American Physiological Society, 1946–1947; now at the University of Oregon Medical School, Portland 1, Oregon.

ml. eggs/liter sea water. The pH of the sea water surrounding the eggs varied from 8.0 to 8.2 throughout the duration of each experiment, and the temperature of the egg suspensions was maintained at 15 ± 0.1° C., except that suspensions of *Lytechinus pictus* eggs were maintained at 20° to 21° C.

The eggs were kept suspended by using a stirrer rotated at 50 r.p.m. P^{32} of high specific activity was added directly to the egg suspensions. The initial concentration of orthophosphate in the suspension fluid, after addition of P^{32}, varied from 50 to 434 μg P/liter (see Protocols). When thoroughly mixed, the homogeneous egg suspension was divided into two lots, one of which was inseminated by adding one drop (0.05 ml.) of solid sperm, directly removed from the testis, to 1000 ml. of suspension. Examination of the eggs shortly after insemination showed approximately two to three spermatozoa at the periphery of each egg. The remainder of the experiment consisted of removing samples of both unfertilized and fertilized eggs at frequent intervals. At the completion of the experiment, the

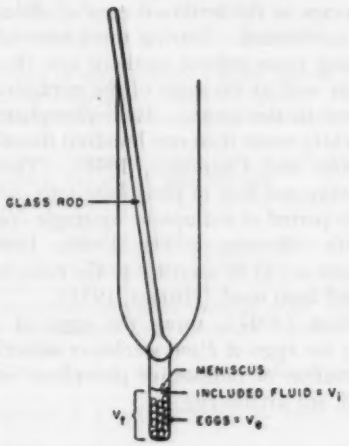

FIGURE. 1. Diagram of Hopkin's tube containing sample of eggs.

eggs previously left unfertilized were inseminated. Development of these eggs, as well as those inseminated earlier, was followed, without diluting the egg suspension, through the pluteus stage for the sea urchin eggs and the trochophore stage for *Urechis* eggs. Development in every case was normal as compared to controls in sea water. This indicated that the P^{32} had been used in concentrations, of the order of 1 to 30 μc/liter, which were below the toxic level.

All experiments were rejected in which (1) the time from insemination to 50 per cent cleavage deviated appreciably from normal, (2) there was undue prolongation of the period between the time when the eggs first started to cleave to the time when cleavage was completed, and (3) less than 95 per cent of the eggs developed to normal swimming embryos.

Removal of egg samples for radioactivity measurements. Each sample of eggs was taken by drawing up one- to two-ml. quantities of the well stirred homogeneous

suspension into a large bore pipette with a wide mouth, and depositing the aliquots in a Hopkin's vaccine tube (Fig. 1) up to the 10.0-ml. mark. The tube was then centrifuged at $86 \times g$ for 60 seconds in a hand centrifuge. This force was just sufficient to drive the eggs into the narrow end of the tube. Within 30 seconds the supernatant was decanted and the fluid remaining within the narrow prolongation of the Hopkin's tube drawn off to a level just above the eggs, using a capillary pipette. The tube was then immediately inverted, and the walls dried with filter paper. The "end point" of penetration of isotope into the eggs was taken as being at the end of the 60 seconds' centrifugation.

The total volume (V_t) of the eggs together with the suspension fluid contained within the narrow prolongation was then determined (Fig. 1). This volume (V_t) amounted to 0.03 to 0.05 ml. in the different experiments. The Hopkin's tube was fixed in a holder fastened to the mechanical stage of a horizontally placed low power microscope provided with an ocular hair line. By operating the stage, the level of the meniscus could be read on the stage scale. Since each tube had been previously accurately calibrated with mercury, the stage scale readings could be converted directly into volume.

After completion of the reading, a thin glass rod with rounded ends was inserted into the tube in order to seal off the mouth of the narrow prolongation (Fig. 1). By holding the rod in place with the index finger, any radioactive solution adhering to the upper walls of the tube was washed out with distilled water without disturbing the eggs at the bottom. After removing the rod, the eggs, together with washings from the bottom of the tube, were transferred to a flat nickel dish 3 cm. in diameter and 3 mm. deep, and dried. The dried material formed a thin even layer on the bottom of the dish, amounting to no more than 1 mg. solids/cm.^{2} of surface. The radioactivity was measured using a Geiger-Müller tube, having a thin mica window 8 cm. in diameter. Samples of the decanted supernatant fluid were dried in the identical dishes and the radioactivity measured.

The question arose as to how accurately the 10.0-ml. aliquots represent the suspension as a whole. This was determined by taking a batch of unfertilized eggs and removing the jelly by several washings. A dozen 10.0-ml. samples were taken as above described in the Hopkin's tubes and centrifuged for ten minutes at $2000 \times g$. The top of the eggs packed in the narrow prolongation of the Hopkin's tubes formed a perfectly straight line, and its level was measured as previously described. The volumes thus obtained were within a maximum range of 0.2 per cent of each other, indicating the validity of the sampling procedure used.

The advantage of the above described method for determining the quantity of radioactive isotope in the eggs is that, by eliminating the necessity for washing the cells, errors which might arise from injury to the eggs and from outward leaching of ions or compounds are avoided.

Egg volume measurements. The mean diameter of fertilized eggs in the early one-cell stage was determined by averaging 25 individual measurements made with a filar micrometer. The unfertilized eggs of the sea urchin are never spherical when freshly removed from the ovaries, and *Urechis* eggs in the unfertilized state are indented on one side. Soon after fertilization the eggs of both species become spherical with only slight changes in volume (Tyler, 1932). The average diameter of *S. purpuratus* eggs is 81.3 μ, *S. franciscanus* eggs 119 μ, and *Urechis caupo* eggs

110 μ. The number of eggs per ml. of suspension was determined as follows. Using a wide-mouthed pipette a sample, approximately 0.2 ml. in volume, was withdrawn from the homogeneous suspension, deposited on a ruled slide, covered with a coverslip, and weighed in order that the volume of the sample could be accurately determined. The total number of eggs on the slide was then counted. This procedure was repeated twice and the results averaged. Knowing the number of eggs in a given mass of sea water and the average diameter of one egg, the total volume of egg protoplasm (Ve) in a 10.0-ml. volume of suspension could be readily calculated.

Egg volume determinations were also carried out by centrifuging the jelly-free unfertilized eggs for 10 minutes at $2000 \times g$. Results obtained by this method were not significantly different from volume determinations carried out by counting the number of eggs in aliquots and measuring diameters.

Calculations. The concentration of P^{32} within the eggs was calculated as follows. Knowing the total volume of eggs with included fluid (V_t) contained within the narrow prolongation of the Hopkin's tube (Fig. 1), and the volume of eggs in 10.0 ml. of suspension (Ve), the volume of the included fluid alone is: $(V_i) = (V_t) - (Ve)$. The included fluid volume (V_i) represents the quantity of fluid exterior to the protoplasmic surface of the eggs. This volume, therefore, includes the space occupied by the egg jelly and the space enclosed within the fertilization membrane, structures which allow free diffusion of phosphate ions. The radioactivity of the included fluid is obtained by multiplying $(V_i) \times$ C.P.M. (counts per minute) of one ml. suspension fluid. Since the radioactivity of the total sample is known, the radioactivity of one ml. of eggs is:

$$\text{C.P.M./ml. eggs} = \frac{(\text{C.P.M. of sample}) - (\text{C.P.M. of included fluid})}{V_e}. \quad (1)$$

Under the conditions of the counting method used, 1 C.P.M. $= 3.6 \times 10^{-6}$ μc P^{32}. Using this conversion factor, the results have been expressed in terms of μc P^{32}/ml. eggs.

Accuracy of method. The following sources of error were taken into consideration in calculating the standard deviation for each determination of the μc P^{32}/ml. eggs (see Tables I and II): the sampling error, error in determining the egg volume (V_e) and the included fluid volume (V_i), error in determining the C.P.M. of the sample, of the background, and of the supernatant fluid. When the radioactivity contributed by the included fluid in each sample is more than half that of the entire sample, the error of the method is considerable. Accuate results are obtained when the concentration of isotope in the eggs exceeds the concentration of isotope in the suspension fluid.

Protocols

Experiment 1. P^{32} was added to a suspension containing 1.00 ml. *S. purpuratus* eggs/liter 120 minutes after the eggs had been removed from the ovaries, and the eggs were inseminated five minutes later. The initial concentration of P^{32} in the sea water was 1.22 μc/liter, and the orthophosphate concentration approximately 71 μg P/liter. First cleavage started 97 minutes after insemination, was 50 per cent complete at 104 minutes and finished at 110 minutes.

Experiment 2. P^{32} was added to a suspension containing 1.09 ml. *S. purpuratus* eggs/liter 135 minutes after removal of the eggs from the ovaries, the eggs being inseminated 12 minutes later. The initial concentration of P^{32} in the sea water

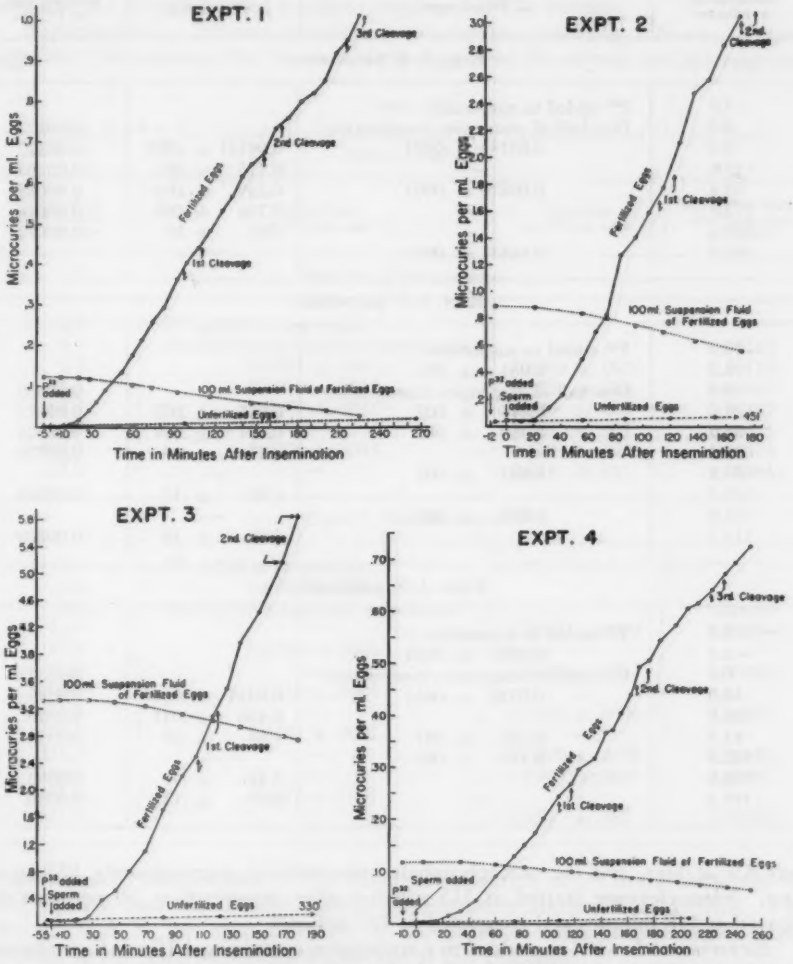

FIGURE 2. Uptake of P^{32} by unfertilized and fertilized sea urchin eggs. Expts. 1, 2 and 3: *S. purpuratus eggs.* Expt. 4: *S. franciscanus eggs.* At beginning of each experiment P^{32} added to suspension and then divided into two lots. Sperm added to one lot at 0 minutes in all experiments—fertilized eggs. The second lot was not inseminated—unfertilized eggs. Ordinates: μc P^{32}/ml. eggs. Abscissae: Time in minutes before and after insemination, 0 = time of insemination. (○——○) fertilized eggs, (●----●) unfertilized eggs, (□····□) μc P^{32}/100 ml. suspension fluid of fertilized eggs.

TABLE I

Uptake of P^{32} by the unfertilized and fertilized eggs of S. purpuratus. Expts. 1, 2, and 3

Time after insemination in minutes	Unfertilized, μc P^{32}/ml. eggs	Fertilized, μc P^{32}/ml. eggs	Suspension fluid of fertilized eggs, μc P^{32}/ml.
	Expt. 1, *S. purpuratus*		
−5.0	P^{32} added to suspension		
0.0	One-half of suspension inseminated		0.00122
8.3	0.00184 ± .0009	0.00111 ± .0009	0.00122
59.6	—	0.175 ± .003	0.00103
92.4	0.00810 ± .0009	0.359 ± .004	0.00085
173.8	—	0.756 ± .006	0.00048
225.1	—	1.01 ± .01	0.00024
266.5	0.0188 ± .0010	—	—
	Expt. 2, *S. purpuratus*		
−12.0	P^{32} added to suspension		
−8.2	0.034 ± .002		
0.0	One-half of suspension inseminated		0.00885
21.0	0.046 ± .002	0.047 ± .003	0.00872
56.0	0.050 ± .002	0.494 ± .009	0.00826
84.4	—	1.27 ± .09	0.00770
87.1	0.063 ± .002	—	—
127.2	—	2.09 ± .14	0.00665
152.0	0.069 ± .003	—	—
171.1	—	3.02 ± .16	0.00560
	Expt. 3, *S. purpuratus*		
−5.5	P^{32} added to suspension		
−2.5	0.0809 ± .0064		
0.0	One-half of suspension inseminated		0.0335
18.0	0.0924 ± .0061	0.0444 ± .0044	0.0333
46.6	—	0.496 ± .071	0.0328
81.5	0.102 ± .007	1.68 ± .09	0.0316
122.5	0.119 ± .006	—	—
128.0	—	3.46 ± .10	0.0294
179.2	—	5.79 ± .12	0.0270

was 8.9 μc/liter, and the orthophosphate concentration approximately 133 μg P/liter. First cleavage started at 113 minutes after insemination, 50 per cent had cleaved at 118 minutes and completed at 123 minutes.

Experiment 3. P^{32} was added to a suspension containing 1.10 ml. *S. purpuratus* eggs/liter 127 minutes after the eggs had been removed from the ovaries, and the eggs were inseminated 5.5 minutes later. The initial concentration of P^{32} in the sea water was 33.5 μc/liter and the orthophosphate concentration approximately 434 μg P/liter. First cleavage started 107 minutes after insemination, was 50 per cent complete at 114.5 minutes and was finished at 121 minutes.

Experiment 4. P^{32} was added to a suspension containing 0.72 ml. *S. franciscanus*

eggs/liter 120 minutes after the eggs had been removed from the ovaries, and the eggs inseminated 10 minutes later. The initial concentration of P^{32} in the sea water was 1.2 μc/liter, and the orthophosphate concentration approximately 59 μg P/liter. First cleavage started 108 minutes after insemination, was 50 per cent complete at 113 minutes and reached completion at 117 minutes.

Experiment 5. P^{32} was added to a suspension containing 1.29 ml. *U. caupo* eggs/liter 180 minutes after removal of the eggs from the animal, and the eggs were

TABLE II

Uptake of P^{32} by the unfertilized and fertilized eggs of S. franciscanus and Urechis caupo. Expts. 4 and 5

Time after insemination in minutes	Unfertilized, μc P^{32}/ml. eggs	Fertilized, μc P^{32}/ml. eggs	Suspension fluid of fertilized eggs, μc P^{32}/ml.
	Expt. 4, *S. franciscanus*		
−10.0	P^{32} added to suspension		
0.0	One-half of suspension inseminated		0.00118
6.3	0.0031 ± .0013	0.0012 ± .0012	0.00118
74.8	0.0038 ± .0014	0.127 ± .005	0.00109
108.2	0.0075 ± .0015	0.240 ± .005	0.00100
163.2	—	0.435 ± .007	0.00086
206.7	0.0094 ± .0015	0.600 ± .009	0.00076
252.0	—	0.718 ± .011	0.00066
	Expt. 5, *Urechis caupo*		
−10.0	P^{32} added to suspension		
−5.0	0.0007 ± .0004		
0.0	One-half of suspension inseminated		0.00089
33.3	—	0.0028 ± .0005	0.00089
43.0	0.0039 ± .0006	—	—
91.5	—	0.0066 ± .0005	0.00088
100.8	0.0056 ± .0006	—	—
176.9	—	0.0145 ± .0006	0.00087
184.1	0.0103 ± .0007	—	—
223.2	—	0.0220 ± .0007	0.00085
266.0	—	0.0369 ± .0010	0.00083
275.0	0.0174 ± .0007	—	—
517.0	—	0.285 ± .005	0.00052

inseminated 10 minutes later. The initial concentration of P^{32} in the sea water was 0.89 μc/liter, and the orthophosphate concentration about 50 μg P/liter. First cleavage was 50 per cent complete at 119 minutes after insemination, second cleavage at 160 minutes and third cleavage at 209 minutes.

RESULTS

Uptake of P^{32} by unfertilized and fertilized eggs

The results of five experiments are presented in Figures 2 and 3. The data are abbreviated in Tables I and II, in which only a few of the determinations are

presented for each experiment. The first column in each table indicates the time when samples of unfertilized and fertilized eggs were removed for radioactivity measurements. P^{32} was added to the suspension of the unfertilized eggs at the beginning of the experiment. Shortly thereafter the first sample was removed, the suspension divided into two lots, and one lot inseminated. In every case the time of insemination is set as zero time. In the second column the quantity of P^{32} which has penetrated the unfertilized eggs after various time intervals is shown. The third column presents the same data for the fertilized eggs. In the fourth column the decrease in concentration of P^{32} in the suspension medium is shown.

Eggs of Strongylocentrotus purpuratus and S. franciscanus. The uptake of P^{32} by the unfertilized eggs is shown in Figure 2, interrupted line with solid circles.

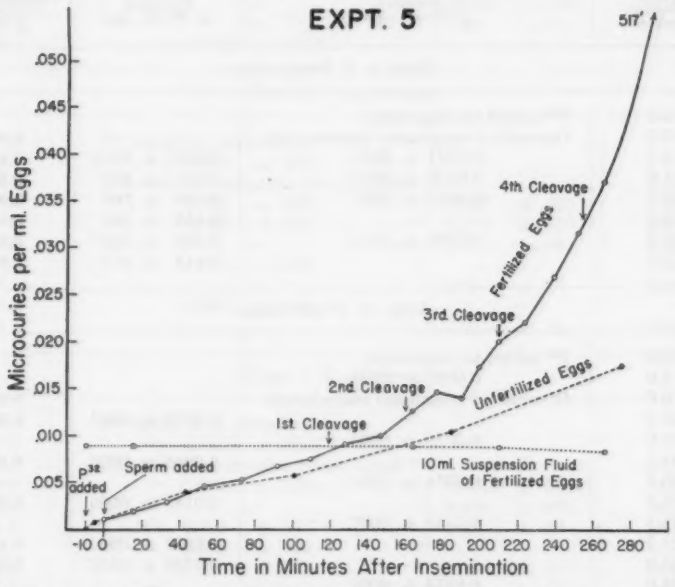

FIGURE 3. Uptake of P^{32} by the unfertilized and fertilized eggs of *Urechis caupo*, Expt. 5. Legend as for Figure 2.

In Experiments 2 and 3 the initial uptake of P^{32}, during the first three to four minutes after adding the isotope, appears to be greater than the uptake in the subsequent 200 minutes. This initial increase, however, undoubtedly does not represent penetration of P^{32} into the eggs, but is an artifact arising from a small error in determining the absolute volume of the eggs, or from the initial absorption of a small quantity of the isotope to the extraneous coats or the surface of the eggs. In Experiments 1 and 4, in which the concentrations of P (as orthophosphate) and P^{32} in the suspension fluid were much less than in Experiments 2 and 3 (see Protocols), the absence of an initial phase of rapid P^{32} uptake is evident. In all

experiments, with the exception of the initial phase in Experiments 2 and 3, the uptake of P^{32} by the unfertilized eggs occurred at a constant rate.

The uptake of P^{32} by fertilized eggs (Fig. 2, solid line with open circles) during the first 10 to 15 minutes following fertilization is identical to the uptake shown by unfertilized eggs. By 15 to 20 minutes following fertilization the rate of uptake increases until by 50 to 60 minutes in *S. purpuratus*, and 80 to 90 minutes in *S. franciscanus* the uptake has reached a maximum rate. Thereafter, except for minor variations, the uptake occurs at a constant rate through the third cleavage. The minor variations in the rate of uptake which occurred were within the range of experimental error. No change in rate of uptake during the cleavage cycles was evident.

Results obtained using the eggs of *Lytechinus pictus* are essentially identical to those obtained using the eggs of the two species of *Strongylocentrotus* (Chambers and Whiteley in Whiteley, 1949).

Eggs of Urechis caupo. The uptake of P^{32} by the unfertilized eggs (Fig. 3, interrupted line with solid circles) was observed to occur at a slow constant rate. The uptake of P^{32} by the fertilized eggs (Fig. 3, solid line with open circles) was essentially identical to that of the unfertilized eggs throughout the period of maturation and the first two cleavages (the eggs, laid in the germinal vesicle stage, do not mature until after insemination occurs). Shortly after the second cleavage the rate at which the fertilized eggs removed P^{32} from the medium increased. Even after the fourth cleavage the rate continued to increase.

Loss of P^{32} from the eggs

Experiments were carried out to determine whether or not P^{32} contained within the eggs is lost to the medium when the P^{32} in the sea water surrounding the eggs is removed. The experiments were carried out using the eggs of *Lytechinus pictus* as follows: Unfertilized and fertilized eggs were exposed to sea water containing P^{32} and approximately 60 μg P as orthophosphate/liter for one hour. Samples of the suspension were then taken to determine the quantity of P^{32} which had entered the eggs, and immediately thereafter the remainder of the suspension was gently centrifuged, the supernatant decanted, and replaced by fresh non-radioactive sea water containing about 60 μg P as orthophosphate/liter. After washing three times by centrifugation, a suspension of the washed eggs was prepared containing 3.0 ml. eggs/liter sea water. Ten-ml. samples of this suspension were removed at various intervals of time for radioactivity determinations. The washing of the eggs was repeated at frequent intervals, in order to remove any P^{32} which may have entered the medium from the eggs. The results are shown in Figure 4. The quantity of P^{32} remaining within the eggs is expressed in terms of the per cent of the quantity of P^{32} within the eggs immediately preceding the first washing. The times when the eggs were washed are indicated by the small arrows in the figures. During careful washing of the eggs, in spite of the greatest precautions it is impossible to avoid destroying or losing some of the eggs. This is particularly true of the fragile unfertilized eggs. Accordingly, the volume of unfertilized eggs in each 10-ml. sample taken was determined by the centrifugal method, and correction made for any loss of eggs which may have occurred during the repeated washings.

The results obtained on the unfertilized eggs (Fig. 4, curves in upper half) reveal that in two experiments 15 per cent, and in one experiment 4 per cent of the P^{32} initially contained within the eggs was lost to the medium during the first 100 minutes after washing was started. The P^{32} continues to be lost at a slow rate over a long period of time. However, the results obtained after 300 minutes from the time the eggs were first washed are open to question, because fertilization and development of these eggs were impaired.

The fertilized eggs are far more resistant to the washing procedure, since they are protected by their fertilization membranes. It was not possible, however, to correct for such losses of eggs as may have occurred, during washing, as egg volumes

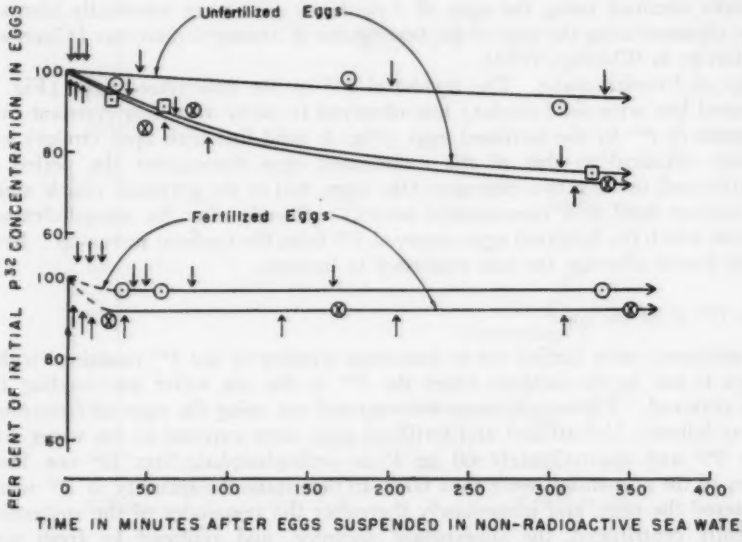

FIGURE 4. Effect of washing unfertilized and fertilized *Lytechinus pictus* eggs containing P^{32} in non-radioactive sea water.

in the samples cannot be determined when the eggs are fertilized. The results of two experiments on fertilized eggs are shown in Figure 4 (curves in lower half). A loss of P^{32} from the samples taken after the first series of washings occurred, but subsequently, no appreciable loss of P^{32} from the eggs was observed. The initial loss of P^{32} is undoubtedly due to the loss of some eggs, for which no correction could be made.

In six experiments carried out using unfertilized and fertilized *S. purpuratus* eggs, similar results were obtained.

The influence of extraneous coats on the uptake of P^{32}

The uptake of P^{32} by the fertilized eggs of *Lytechinus pictus* from which the extraneous coats had been removed was compared to that of normal eggs possessing

all their coats intact. The experiment was performed as follows: A suspension of unfertilized eggs was divided in two beakers. The eggs in one beaker were inseminated. One-half of this suspension was centrifuged, the supernatant discarded and two minutes after insemination, at the time when observation revealed that the fertilization membranes were rising, the eggs were suspended in a mixture of 95 parts 1 *M* urea, pH 8.0, and five parts sea water. The eggs were allowed to settle, and five minutes after insemination the urea solution was decanted and replaced by sea water. The decantations were repeated several times until the eggs had been washed free of the urea solution. Observation of the eggs revealed that the jelly coats and the fertilization membranes had been completely removed and that the hyaline plasma layer did not form (Moore, 1930). When the urea-treated eggs cleaved, they separated into two spherical blastomeres, fastened together only by delicate stalks. The fertilized untreated eggs and the urea-treated eggs cleaved 100 per cent and at the same time. At 50 minutes after insemination, P^{32} was added to all three suspensions, the control unfertilized eggs, the control fertilized eggs, and the urea-treated fertilized eggs. Samples were removed at intervals for radioactivity determinations. The results are shown in Table III. They reveal that in the 25-minute interval during which the uptake of P^{32} was measured, 75 times as much P^{32} entered the fertilized eggs as the unfertilized eggs,

TABLE III

Uptake of P^{32} by unfertilized, normal fertilized and urea-treated fertilized Lytechinus pictus eggs

Condition of eggs	Uptake of P^{32}, in C.P.M./ml. eggs
Unfertilized	2,000
Fertilized, controls	150,000
Fertilized, urea treated	145,000

and that as much P^{32} entered the denuded urea-treated eggs as the control fertilized eggs. Since the urea-treated eggs in sea water may still have possessed a thin but invisible coating of proteinaceous material, at the end of the 25-minute interval these eggs were washed for 10 minutes in three changes of an isotonic mixture of 10 parts 0.53 *M* KCl and 90 parts 0.52 *M* NaCl at pH 6.0 and then suspended in this mixture. Such a treatment should have dissolved away any remaining extraneous material surrounding the eggs (Chambers, 1940). The washing of the urea-treated eggs in the Na/K mixture did not remove an appreciable quantity of P^{32} from the eggs, revealing that no significant quantity of P^{32} is absorbed to the coats, which surround the eggs external to the protoplasmic surface.

Rate of penetration into the eggs

The quantity of P^{32} in μc entering one ml. eggs in a given interval of time, $t_2 - t_1$, may be obtained directly from the graphs. Rates of penetration were determined only for the constant phases of uptake, *i.e.*, from five minutes (t_1) to 200 minutes (t_2) after addition of P^{32} for the unfertilized eggs, from 70 minutes (t_1) to 170 minutes (t_2) after insemination for the fertilized sea urchin eggs, and from 250 minutes (t_1) to 275 minutes (t_2) after insemination for the fertilized *Urechis* eggs.

In spite of a very considerable decrease in the concentration of P^{32} in the medium

surrounding the fertilized sea urchin eggs, the uptake of P^{32} by the eggs remained constant throughout the duration of Experiments 1–4. In experiments on the unfertilized eggs, and the fertilized eggs of *Urechis caupo*, no appreciable change in concentration of P^{32} in the suspension fluid occurred, since only a small quantity of P^{32} penetrated the eggs.

Fertilized eggs of S. purpuratus and S. franciscanus. The decrease in concentration of P^{32} in the medium surrounding the fertilized eggs could be due either to an exchange of P^{32} from the external medium for P inside the eggs, or to an accumulation of P within the eggs, depleting the P in the suspension fluid. Experiments described in this paper reveal that when fertilized eggs, which had been exposed to sea water containing P^{32}, are immersed in radioactive-free sea water containing about 60 μg P/liter no appreciable quantity of P^{32} leaves the eggs. Chambers and White (1949, 1954) have shown that fertilized eggs remove orthophosphate from a medium containing between 10 to 100 μg P/liter at the same rate as the P^{32}.

The specific activity (μc P^{32}/μg P) of the P (as orthophosphate) in the medium, therefore, remains constant throughout the duration of the experiment. Accordingly, the rate of penetration of orthophosphate into the eggs can be accurately calculated as follows:

$$\mu\text{g P/ml. eggs/min.} = \left(\frac{\mu\text{g } P_s}{\mu\text{c } P_s^{32}}\right)\left(\frac{\mu\text{c } P_e^{32} \text{ at } t_2 - \mu\text{c } P_e^{32} \text{ at } t_1}{(t_2 - t_1)}\right), \qquad (2)$$

where μg P_s = initial concentration of orthophosphate in the suspension fluid in μg P/ml., μc P_s^{32} = initial concentration of P^{32} in μc/ml. in the suspension fluid, μc P_e^{32} at t_2 and t_1 = concentration of P^{32} in μc/ml. eggs at the time in minutes t_2 and t_1.

The rates of penetration of orthophosphate, calculated according to equation (2) are shown in Table IV, including three experiments from Chambers and White (1954.)

For the fertilized eggs of *S. purpuratus*, the rate of P uptake, from 70 to 170 minutes after insemination, at 15° C., in four experiments, varied from 0.54 μg P/ml. fertilized eggs/minute at an external orthophosphate concentration of 416 μg P/liter to 0.28 μg P/ml. fertilized eggs/minute at an external orthophosphate concentration of 20 μg (P/liter (Table IV, columns 3 and 5). With a twenty-fold change in concentration of P, only a 1.9-fold change in the rate of penetration of P occurred. As long as the orthophosphate concentration exceeds about 20 μg P/liter, the more dilute the orthophosphate concentration, the greater is the fraction of P in the medium which is absorbed by the fertilized eggs in a given period of time.

The rate of penetration of orthophosphate into fertilized *Strongylocentrotus franciscanus* eggs from 70 to 170 minutes after insemination, at 15° C., in three experiments, ranged from 0.11 to 0.17 μg P/ml. fertilized eggs/minute, with the concentration of orthophosphate in the external medium varying from 63 to 20 μg P/liter (Table IV, columns 3 and 5). The rate of uptake by the fertilized *S. franciscanus* eggs is about half that of *S. purpuratus* eggs.

Unfertilized eggs of S. purpuratus and S. franciscanus. In the experiments carried out with unfertilized eggs, no change could be detected in the concentration

of P^{32} in the suspension fluid. Although the concentration of orthophosphate in the sea water surrounding unfertilized sea urchin eggs remains constant or slowly increases (Chambers and White, 1954), the amount of increase is not sufficient to appreciably alter the specific activity of the orthophosphate in the medium as long as the egg suspension is dilute (1.0 ml. eggs/liter suspension) and the concentration of orthophosphate in the medium exceeds 50 μg P/liter.

Unlike fertilized eggs, unfertilized eggs containing P^{32}, when immersed in non-radioactive sea water, slowly lose P^{32} to the surrounding medium. The rate, however, at which P^{32} is lost from eggs which had been exposed to P^{32} sea water is over a hundred times slower than the rate at which the P^{32} originally entered the eggs. In view of these considerations and the linear P^{32} uptake curves, equation

TABLE IV

Rate of penetration of phosphate into unfertilized and fertilized S. purpuratus, S. franciscanus and Urechis caupo eggs at 15° C.

Experiment	Condition of eggs	Initial and final P conc. μg P/liter susp. fluid	μg P/ml. eggs/min.		Fertilized, μg P/ml. eggs/min. Unfertilized, μg P/ml. eggs/min.
			Unfertilized	Fertilized	
S. purpuratus,	Unfert.	71	0.0035		
Expt. 1	Fert.	56 to 29		0.30	86
S. purpuratus,	Unfert.	133	0.0035		
Expt. 2	Fert.	122 to 84		0.37	106
S. purpuratus,	Unfert.	434	0.0041		
Expt. 3	Fert.	416 to 359		0.54	132
*S. purpuratus**	Unfert.	78	0.0026		
	Fert.	78 to 20		0.28	106
S. franciscanus,	Unfert.	59	0.0015		
Expt. 4	Fert.	55 to 42		0.17	113
S. franciscanus†	Fert.	53 to 20		0.11	—
S. franciscanus‡	Fert.	63 to 20		0.14 ot 0.17	—
U. caupo,	Unfert.	50	0.0033		
Expt. 5	Fert.§	47 to 46		0.024	7

* From Chambers and White (1954), Expts. 5 and 6.
† From Chambers and White (1954), Expt. 7.
‡ From Chambers and White (1954), Expt. 8.
§ After third cleavage.

(2) may be used to calculate the rate of entry of orthophosphate into unfertilized sea urchin eggs.

In the case of the unfertilized eggs of *S. purpuratus* the rate of penetration of orthophosphate at 15° C. in three experiments varied between 0.0041 to 0.0026 μg P/ml. unfertilized eggs/minute with concentration of P (as orthophosphate) in the medium ranging from 434 to 71 μg P/liter (Table IV, columns 3 and 4). For the unfertilized eggs of *S. franciscanus* the rate of P uptake, in one experiment, was 0.0015 μg P/ml. unfertilized eggs/minute, at an external orthophosphate concentration of 59 μg P/liter.

Fertilized and unfertilized Strongylocentrotus eggs compared. As shown in Table IV, column 6, phosphate penetrates fertilized *S. purpuratus* eggs 86 to 132 times more rapidly than unfertilized eggs.

In *S. franciscanus* eggs, approximately a 113-fold increase in rate occurs after fertilization.

Eggs of Urechis caupo. The rates of penetration of orthophosphate into the unfertilized and fertilized eggs were arbitrarily calculated according to equation (2). In view of the slow rate of P^{32} uptake, the inappreciable change in concentration of P^{32} in the suspension fluid, and the dilute egg suspension, it is probable that the use of equation (2) is justified.

Phosphate penetrates the unfertilized eggs of *Urechis caupo* and *S. purpuratus* at about the same rate (Table IV, columns 3 and 4). The important difference between the *Urechis* and the sea urchin egg is that in the former species, after fertilization, no increase in the rate of P uptake occurs. After the *Urechis* eggs have undergone second cleavage, however, the rate starts to increase, attaining a rate 7 times that of the unfertilized eggs by the time of fourth cleavage (Table IV, column 6). Even by this time the rate has not reached its maximal level (Fig. 3).

Discussion

When fertilized sea urchin eggs which had been exposed to sea water containing P^{32} are immersed in radioactive free sea water, no appreciable quantity of P^{32} leaves the eggs. Furthermore, fertilized sea urchin eggs remove orthophosphate from sea water at the same rate as P^{32} (Chambers and White, 1949, 1954). These data reveal that the entry of P^{32} into the fertilized sea urchin eggs measures the rate at which phosphate accumulates within the cells.

On the other hand, when P^{32} is added to a suspension of unfertilized eggs, no appreciable change in concentration of P^{32} occurs in the suspension fluid. The concentration of orthophosphate in the suspension fluid surrounding unfertilized eggs remains constant or slowly increases (Chambers and White, 1954). When unfertilized eggs, containing P^{32}, are immersed in non-radioactive sea water, P^{32} slowly washes out into the external medium. On the basis of these data, the conclusion may be made that the uptake of P^{32} by unfertilized eggs measures the rate at which phosphate enters the eggs, presumably by exchange, at the same time that the internal concentration remains constant, or even decreases. The change from the unfertilized to the fertilized state, therefore, involves not only a change in magnitude but also a reversal of "driving forces." Although the two-fold increase following fertilization in permeability to water (Lillie, 1916) and non-electrolytes (Stewart and Jacobs, 1932) may contribute to the striking increase in uptake of orthophosphate which follows fertilization, it is probable that changes in "driving forces" play the dominant role.

Of interest is the relatively constant rate at which phosphate accumulates within fertilized sea urchin eggs, irrespective of large changes in concentration in the external medium. This resembles the constancy in the rate of oxygen consumption of cells, over a wide range of different oxygen tensions, as long as the tension exceeds a certain minimal value. Apparently the primary factor which determines the rate of phosphate entry into fertilized eggs is the rate at which phosphate is bound or combined within the cells.

The question arises as to the importance of phosphate in sea water for the development of the eggs. Both Loeb (1907) and Herbst (1898) reached the conclusion that phosphate in the medium is not necessary for normal development.

However, these investigators used artificial sea water prepared from the individual salts, which had not been specially purified, and the only criterion for the absence of phosphate was the lack of a positive test with a molybdate method which was far too insensitive. Herbst used highly dilute sea urchin egg suspensions in the order of several drops of eggs to a beaker of sea water. In view of the data presented in this paper, under such conditions even a trace of phosphate would have been sufficient to adequately supply the eggs. Eggs with a very low intracellular inorganic phosphate content, such as the eggs of *S. purpuratus* (Chambers and White, 1949) and *S. dröbachiensis* (Chambers and Mende, 1953a, 1953b), may have more need for an external source of phosphate than eggs with a high content of inorganic phosphate, such as *Arbacia punctulata* (Chambers and Mende, 1953b), and *Paracentrotus lividus* (Zielinski, 1939).

On the basis of experiments in which the rate of P^{32} uptake by *S. purpuratus* spermatozoa was measured in a suspension containing 1.0 ml. solid sperm/liter sea water, it was found that one ml. of solid sperm takes up P^{32} more slowly than a corresponding volume of unfertilized eggs (Chambers and White, unpublished data). Accordingly, the amount of P^{32} which would enter the few excess spermatozoa attached to the outer surface of fertilized eggs is infinitesimal, compared to the amount actually found to enter the eggs.

Relation to oxygen consumption. The slow rate of P^{32} uptake by unfertilized sea urchin eggs (two to six hours after removal from the ovaries) is observed during the period when the eggs would show a fairly constant and low rate of oxygen consumption (Borei, 1948, 1949). The prominent increase in the rate of uptake of P^{32}, which occurs after sea urchin eggs are inseminated and represents the accumulation of phosphate within the fertilized eggs, takes place during a period when the rate of oxygen consumption increases markedly (Borei, 1948; Tyler and Humason, 1937).

Laser and Rothschild (1939) describe a marked increase in the rate of oxygen consumption of *Psammechinus miliaris* (sea urchin) eggs at 20° C. within the first five minutes after insemination, followed by a fall to the original unfertilized level within ten minutes. During the corresponding period in the eggs of sea urchins, no increase in the rate of penetration of orthophosphate was observed. However, within the first 6 to 7 minutes after insemination at 16° C., a prominent decrease in the concentration of intracellular inorganic phosphate has been noted in the eggs of *S. purpuratus* (Chambers and White, 1949) and *S. dröbachiensis* (Chambers and Mende, 1953b).

The rate of oxygen consumption of sea urchin eggs reaches a maximum some time before first cleavage (Runnström, 1933) and remains fairly constant during the next several cleavages. The rate of P^{32} uptake (at 15° C., for both species of *Strongylocentrotus* eggs) reaches a maximum between 30 to 40 minutes before first cleavage and remains constant thereafter through the first two or three cleavage cycles.

Zeuthen (1949, 1950a, 1950b, 1951) has demonstrated that superimposed on the basic oxygen consumption curves of sea urchin eggs are definite waves of relatively small magnitude, the minima corresponding to the periods of cytoplasmic division, the maxima to the prophases. Although no alterations could be detected

in the rate of P^{32} uptake during the first few cleavages, it should be emphasized that the accuracy of the P^{32} uptake method described in this paper is such that waves of considerably greater magnitude than those described by Zeuthen would not have been detected. However, using a method of much greater accuracy, cyclic variations in the rate of P^{32} uptake have been observed during the later segmentation stages of sand dollar eggs (Chambers, White and Zeuthen in Zeuthen, 1951).

In the experiment carried out using the eggs of *Urechis caupo*, obtained from freshly collected animals, the rate of P^{32} uptake did not increase following fertilization until after the second cleavage. This may be related to the fact that an increase in the rate of oxygen consumption does not occur in these eggs, obtained from freshly collected animals, until during the later cleavage stages (Tyler and Humason, 1937; Horowitz, 1940).

Changes in the rate of oxygen consumption appear to parallel changes in the rate of accumulation of phosphate in the marine eggs studied, at least before and after fertilization and during the early cleavage stages. Following fertilization, the sea urchin eggs accumulate phosphate, since energy from oxidative processes is utilized in the synthesis of organic phosphorous-containing compounds (Chambers and Mende, 1953b).

Summary

1. A method is described for measuring the concentration of radioactive isotope in cells without washing the cells free of the surrounding radioactive medium.

2. P^{32} as orthophosphate penetrates the unfertilized eggs of all species at a slow and constant rate.

3. During the first 10 to 15 minutes following the insemination of *Strongylocentrotus purpuratus* and *S. franciscanus* eggs, the rate of P^{32} uptake is essentially identical to that of the unfertilized eggs. The rate of uptake increases by 15 to 20 minutes, and reaches a maximum by 50 to 90 minutes after insemination. Thereafter, through the first three cleavages the rate remains constant, within the limits of error of the method, as long as the concentration of P in the medium is in excess of 20 μg P/liter.

4. Following insemination of *Urechis caupo* eggs, the rate of P^{32} uptake does not increase. After the second cleavage, however, the rate of P^{32} uptake increases, and a maximum rate has not been attained even after the fourth cleavage.

5. When fertilized eggs containing P^{32} are suspended in non-radioactive sea water, they slowly lose P^{32} to the external medium. On the other hand, no P^{32} is lost from fertilized eggs containing radioactive phosphate when they are washed in non-radioactive sea water.

6. P^{32} is not absorbed to the extraneous coats of fertilized eggs.

7. The rate of penetration of phosphate into the unfertilized and fertilized eggs has been calculated in terms of the μg P entering one ml. eggs/minute. The rate at which phosphate enters fertilized *Strongylocentrotus* eggs is relatively independent of the external concentration, as long as this exceeds 20 μg P/liter. Phosphate enters fertilized *Strongylocentrotus* eggs 86 to 132 times faster that it penetrates the unfertilized eggs.

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THE ACCUMULATION OF PHOSPHATE BY FERTILIZED SEA URCHIN EGGS[1]

EDWARD L. CHAMBERS[2] AND WILLIAM E. WHITE[3]

Department of Zoology, University of California, Berkeley, California

Radioactive phosphate enters fertilized sea urchin eggs far more rapidly than it enters the unfertilized eggs (Brooks and Chambers, 1948, 1954; Abelson, 1947; Lindberg, 1948; Whiteley, 1949). Investigations described in this paper demonstrate that the entry of P^{32} into the eggs represents an accumulation of phosphate within the eggs. In addition, the concentrations of P and P^{32} in the inorganic and organically bound phosphate fractions of the eggs have been measured, with the purpose of determining in which fractions the phosphate, accumulated by the fertilized eggs, is incorporated, and whether the process of accumulation is associated with alterations in the distribution of P within the eggs.

Materials and Methods

Eggs of the Pacific coast sea urchins *Strongylocentrotus purpuratus, S. franciscanus,* and *Lytechinus pictus* were prepared for use, and measurements of egg volume, and of P^{32} concentration in the eggs and suspension fluid carried out as described previously (Brooks and Chambers, 1954). The jelly was removed from the eggs by repeated washings in sea water. The sea water used in the experiments was filtered through fine mesh filter paper. The experiments were performed at $15 \pm 0.1°$ C. unless otherwise stated. The pH of the sea water in which the eggs were suspended was measured at intervals throughout the duration of the experiments, and varied between pH 8.0 to 8.2. Carrier-free P^{32}, as orthophosphate, was added to the egg suspensions in amounts which varied from 0.2 to 2 μc P^{32}/liter of suspension. The concentration of orthophosphate in the sea water was measured using the Deniges-Atkins method (Atkins, 1923) with corrections for reagent blank and salt error (Cooper, 1938).

Trichloroacetic acid extracts of unfertilized and fertilized eggs were prepared as described by Chambers and Mende (1953a). Measurements of the P and P^{32} content of the inorganic and easily hydrolyzable phosphate fractions of the trichloroacetic acid-soluble extracts were carried out using the isobutyl alcohol extraction method of Borbiro and Szent-Györgyi (1949). After measurement of the phosphomolybdate concentration in the isobutyl alcohol extracts, aliquots were pipetted into flat dishes, evaporated, and the P^{32} concentration measured using a Geiger-Müller counter. In all experiments described in this paper samples of the egg

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[2] Now at the University of Oregon Medical School, Portland 1, Oregon.

[3] Now at the University of Kansas Medical School, Kansas City, Kansas.

suspensions, as originally prepared, were kept for observation. If the suspension was of unfertilized eggs, these were inseminated at the completion of the experiment. Over 95 per cent of the eggs in these samples developed to normal swimming gastrulae.

Results

Removal of P and P^{32} from the suspension fluid, and uptake of P^{32} by the eggs in suspensions of sea urchin eggs

Suspensions of unfertilized and fertilized eggs were prepared in filtered sea water containing 2.5 to 5.6 ml. eggs/liter. Small quantities of orthophosphate and P^{32} were added to the suspensions. The initial concentration of orthophosphate in the suspension fluid varied between less than 4 μg to 78 μg P/liter, and the initial concentration of P^{32} from 0.23 to 0.28 μc/liter.

Unfertilized eggs. The results obtained using suspensions of unfertilized *S. purpuratus* eggs are shown in Table I. Measurements of P and P^{32} concentrations were begun two hours after the eggs had been removed from the ovaries. In Experiments 1, 2 and 3 the concentration of P in the external medium increased,

Table I

Concentration of P in the suspension fluid of a suspension of unfertilized S. purpuratus eggs. Experiments 1 to 5

Expt. No.	Ml. eggs/l. suspension	Time between P analyses in minutes	Initial P conc., μg/l. susp. fluid	Final P conc., μg/l. susp. fluid	μg P lost/ml. eggs/min.	μg P entering/ml. eggs/min.
1	5.6	97	38	55	0.032	0.0011
2	4.1	120	8	20	0.024	—
3	4.7	312	30	56	0.019	—
4	5.5	115	4	5	0.0	—
5	2.5	159	78	78	0.0	0.0026

while in Experiments 4 and 5 no appreciable change in the P concentration could be detected (Table I, columns 4, 5 and 6). There was no measurable change in concentration of P^{32} in the suspension fluid in any of the experiments. In Experiments 1 and 5 the uptake of P^{32} by the eggs was measured and the quantity of P entering one ml. eggs/minute calculated (Table I, column 7), as previously described by Brooks and Chambers (1954). The results reveal that P enters unfertilized eggs whether or not the eggs simultaneously lose P to the external medium. At the completion of the experiments the unfertilized eggs were inseminated, and they developed normally through the gastrula stage.

Fertilized eggs. The results obtained using suspensions of fertilized *S. purpuratus* and *S. franciscanus* eggs are shown in Tables II and III, and Figure 1. The eggs were inseminated two hours after removal from the ovaries, washed free of spermatozoa by gentle centrifugation, and suspended in sea water containing known concentrations of orthophosphate and P^{32}.

In Experiment 6 (Table II, Fig. 1) at 20.5 minutes after insemination 2.5 ml. of *S. purpuratus* eggs were suspended in a liter of sea water containing 78 μg P/liter. A prominent decrease in concentration of P and P^{32} in the medium occurred (Expt.

6, Table II, columns 2 and 4, and Fig. 1). The rate of uptake of P^{32} by the eggs (Table II, column 6) was identical to the rate of disappearance of P and of P^{32} from the medium (Table II, compare columns 3, 5 and 7). The initial lag in the disappearance of orthophosphate and of P^{32} from the medium (Fig. 1, Expt. 6, from 0 to 30 minutes) is due to the fact that the uptake of P by fertilized eggs does not reach a maximum until about one hour after insemination (Brooks and Chambers, 1954). Subsequently, orthophosphate is removed from the medium at a constant rate until the concentration falls to 15 to 20 μg P/liter (Fig. 1, Expt. 6). The rate of uptake then falls off sharply.

TABLE II

Concentration of P and P^{32} in the suspension fluid, and of P^{32} in the eggs in suspensions of fertilized eggs.

Experiments 6 and 7

Time after initial measurement in minutes	μg P/l. susp. fluid	Per cent initial P conc. in susp. fluid	CPM P^{32}/l. susp. fluid	Per cent initial P^{32} conc. in susp. fluid	CPM P^{32} in eggs/l. suspension	Per cent initial P^{32} conc. in eggs
Experiment 6. *S. purpuratus* eggs						
0.0*	78	100.0	61,000	100.0	0	0.0
17.5	75	96.0	56,600	92.6	4,780	7.8
60.5	48	61.5	36,780	60.2	24,900	40.8
91.5	22	28.2	16,960	27.8	42,920	70.5
129.5	6	7.7	6,600	10.8	54,480	90.0
213.0	<2	<3.0	3,200	5.2	58,140	95.5
387.5	<2	<3.0	2,360	3.8	—	—
Experiment 7. *S. franciscanus* eggs						
0.0†	53.5	100.0	76,000	100.0	0	0.0
31.0	40.0	74.8	62,000	81.6	15,200	20.0
68.0	27.5	51.4	—	—	—	—
139.0	10.0	18.7	17,160	22.6	57,380	75.4
192.0	5.0	9.3	9,200	12.1	67,020	88.0

* Eggs inseminated 20.5 minutes before initial measurment.
† Eggs inseminated 63 minutes before initial measurement.

In Experiment 7 (Table II, Fig. 1) at 63 minutes after insemination 3.5 ml. of *S. franciscanus* eggs were suspended in a liter of sea water containing 53.5 μg P/liter. The results are similar to those obtained in Experiment 6. The eggs remove orthophosphate from the external medium at a constant rate until the concentration falls below 20 μg P/liter, when the rate of uptake by the eggs falls off sharply (Fig. 1).

In Experiment 8 (Table III) at 43 minutes after insemination 4.9 ml. of *S. franciscanus* eggs were suspended in a liter of sea water. The P and P^{32} concentrations in the suspension fluid were measured at the beginning and at the end of successive 30-minute periods. Additional amounts of P and P^{32} were added to replenish the external medium prior to each 30-minute period. As in the two

previous experiments, the decrease in concentration of P^{32} parallels the decrease in concentration of P in the suspension fluid (Table III, columns 6 and 7). The results show that as long as the concentration of orthophosphate is over 18 to 20 μg P/liter, the rate of uptake of P and P^{32} remains fairly constant during the first 430 minutes after insemination (Table III, column 8).

Distribution of P^{32} between the trichloroacetic acid-soluble and -insoluble fractions of the eggs

Suspensions of *Lytechinus pictus* eggs were prepared containing one ml. eggs/liter of sea water maintained at a temperature of 20 to 21° C. Two hours after

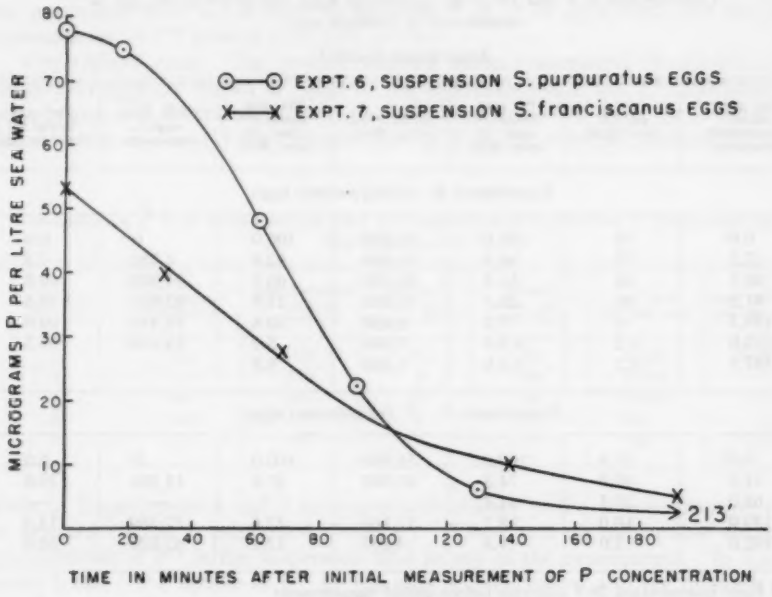

FIGURE 1.

the eggs had been removed from the ovaries, carrier-free P^{32} was added. At various intervals of time duplicate 20-ml. samples of the suspension were removed, the eggs washed three times by centrifugation at $86 \times g$ for three minutes in non-radioactive sea water, the supernatant sea water decanted, an equal volume of ice cold 10 per cent trichloroacetic acid added to each of the duplicate samples, the trichloroacetic acid-soluble and -insoluble fractions separated, and the P^{32} content of the fractions measured.

In unfertilized eggs, between 95.7 to 95.9 per cent of the P^{32} was recovered in the trichloroacetic acid-soluble extracts, with 4.1 to 4.3 per cent in the acid-insoluble fractions, after the eggs had been exposed to P^{32} for two hours.

In the process of washing the unfertilized eggs in non-radioactive sea water

prior to homogenization, from 4 to 8 per cent of the P^{32} initially present in the eggs was removed. The effect of this loss of P^{32} is to decrease the relative proportion of P^{32} contained in the trichloroacetic acid-soluble extracts of the washed eggs by 0.2 to 0.3 per cent.

In experiments carried out using fertilized eggs, the eggs were inseminated two hours after removal from the ovaries and P^{32} was added at the time of insemination. In eggs exposed to P^{32} for a period of 45 to 120 minutes after insemination, and washed for a period of 20 to 30 minutes in non-radioactive sea water, 96.3 to 96.4 per cent of the P^{32} was found in the trichloroacetic acid-soluble extracts, and 3.6 to 3.7 per cent in the acid-insoluble residue. No appreciable quantity of P^{32} is lost from fertilized eggs when washed in non-radioactive sea water (Brooks and Chambers, 1954). The slightly lower proportion of P^{32} in the acid-insoluble fraction of fertilized eggs, as compared to the unfertilized, may be entirely due to the loss of P^{32} from the unfertilized eggs when they are washed prior to homogenization.

TABLE III

Suspension of fertilized S. franciscanus eggs. Disappearance of P and P^{32} from the suspension fluid during thirty-minute periods, following successive additions of P and P^{32}. Experiment 8

Time after insem., in 30 minute intervals	Initial concentration:		Final concentration:		Per cent decrease P conc.	Per cent decrease P^{32} conc.	μg P/ml. eggs/min.
	μg P/l.	CPM P^{32}/l.	μg P/l.	CPM P^{32}/l.			
50 to 80	63.5	598,000	41.0	356,000	35	37	0.15
110 to 140	50.5	473,000	25.7	248,000	49	48	0.17
160 to 190	47.0	—	23.5	—	50	—	0.16
210 to 240	57.0	578,000	35.8	350,000	37	39	0.14
290 to 320	38.5	385,000	18.0	214,000	53	45	0.14
325 to 355	(16.0)	(220,000)	(10.0)	(140,200)	(37)	(35)	(0.04)
400 to 430	57.5	550,000	30.7	286,000	47	48	0.18

In a series of experiments, after exposing the inseminated eggs to P^{32} for 50 minutes and washing, the eggs were allowed to develop 400 minutes in non-radioactive sea water to the early blastula stage prior to homogenization. No P^{32} was lost from the fertilized eggs during the long period of development in the sea water free of P^{32}, even though the medium surrounding the eggs was repeatedly replaced by fresh sea water. The quantity of P^{32} found in the acid-soluble extract amounted to 93.0 per cent of the total, with 7.0 per cent in the acid-insoluble fraction, as compared to 96.4 and 3.6 per cent, respectively, in the corresponding experiment on fertilized eggs homogenized immediately after washing. The experiment reveals that a substantial portion of the phosphate, initially accumulated in the eggs, becomes incorporated in the acid-insoluble fraction. This conclusion is based on the consistency with which a lower percentage of P^{32} was found in the acid-insoluble residue of fertilized eggs continuously exposed to P^{32}.

In the control experiment P^{32} was added only after the eggs had been suspended in trichloroacetic acid. Even after repeated washing of the acid-insoluble residue with trichloroacetic acid, 1.0 per cent of the P^{32} was retained in this fraction. This experiment indicates that the quantity of P^{32} organically combined in the acid-

insoluble residue is probably less by at least one per cent than the quantities actually found.

The distribution of P^{32} between the trichloroacetic acid-soluble and -insoluble fractions of *S. purpuratus* eggs, both unfertilized (four experiments) and fertilized (six experiments) is essentially identical to that found in the eggs of *Lytechinus pictus*.

Lipids and phospholipids were extracted from the acid-insoluble residue of the fertilized *L. pictus* eggs. The acid-insoluble residue, after complete extraction with a mixture of three volumes ethanol and one volume ether, retained 92.1 per cent of the original P^{32} content. The ethanol-ether extract was dried, and the residue extracted with petroleum ether. The petroleum ether fraction containing the phospholipids accounted for 7.5 per cent of the total P^{32} content of the trichloroacetic acid-insoluble fraction. The remaining 0.5 per cent was in the petroleum ether-insoluble fraction.

TABLE IV

Distribution of P and P^{32} in the acid-soluble extracts of Strongylocentrotus purpuratus eggs. Experiments 9, 10 and 11

Expt. No.	Condition of eggs	μg P/ml. eggs ± std. dev.			Per cent total P^{32}:		
		Inorg. P	Labile P	Inorg. + labile P	Inorg. P^{32}	Labile P^{32}	Acid stable P^{32}
9	Unfertilized	58±.6	408±4	466±4	24	66	10
	Fertilized	16±.2	456±5	472±5	6	88	6
10	Unfertilized	69±.7	415±4	484±5	—	—	—
	Fertilized	34±.4	451±5	485±5	8	84	8
11	Unfertilized	77±.8	460±5	537±5	—	—	—
	Fertilized	39±.4	496±5	535±5	9	84	7

Distribution of P and P^{32} in the trichloroacetic acid-soluble extracts of S. purpuratus eggs

The quantities of inorganic P and P liberated after 10 minutes' hydrolysis in 1 N HCl at 100° C. in the trichloroacetic acid-soluble extracts of unfertilized and fertilized eggs were determined. The results of three representative experiments are shown in Table IV, Experiments 9, 10 and 11. Five ml. of *S. purpuratus* eggs were suspended in a liter of sea water containing 20 to 50 μg P as orthophosphate/liter. The suspension was divided into two equal lots. Carrier-free P^{32}, 1 μc/100 ml. suspension, was added to one lot of unfertilized eggs one hour after removal from the ovaries. The other lot was inseminated two hours after the eggs had been removed from the ovaries, and at the same time duplicate 100-ml. samples were removed from the suspension of unfertilized eggs, centrifuged, and the trichloroacetic acid extracts prepared. Thirty minutes after insemination 0.1 μc P^{32}/100 ml. suspension was added to the fertilized eggs, and at 60 minutes after insemination, duplicate 100-ml. samples were removed, the fertilized eggs washed twice in non-radioactive sea water by centrifugation, and the trichloroacetic acid extracts pre-

pared. The results show that following insemination, a prominent decrease in the concentration of inorganic P occurs within the eggs (Table IV, column 3), and at the same time a corresponding increase in the concentration of P liberated after hydrolysis (Table IV, column 4). Within the errors of the measurements, the sum of the inorganic P and P liberated after hydrolysis is the same both before and after insemination (Table IV, column 5).

The distribution of P^{32} between the various P fractions in the trichloroacetic acid extracts is shown in Table IV, columns 6, 7 and 8. The results reveal that the major portion of the P^{32} is associated with easily hydrolyzable organic P compounds. Following insemination, with the accompanying decrease in quantity of inorganic P and the increase in amount of P liberated after hydrolysis, the proportion of P^{32} in the easily hydrolyzable P fraction increases markedly. The proportion of P^{32} in the acid-stable P compounds is small, in spite of the fact that Whiteley (1949) reports the presence, in trichloroacetic acid extracts, of 511 μg acid-stable P/ml. *S. purpuratus* eggs, which amounts to approximately one-half of the total P content in the acid-soluble extract.

Discussion

The experiments presented in this paper establish conclusively that the entry of P^{32} into the fertilized eggs quantitatively measures the accumulation of orthophosphate within the eggs. However, P^{32} probably enters unfertilized eggs by an exchange process, since the quantity of orthophosphate in the medium surrounding the eggs either remains constant, or slowly increases. Measurements of the distribution of P^{32} in the trichloroacetic acid-soluble extracts of the eggs reveal that in both unfertilized and fertilized eggs the P^{32} is confined primarily to the intracellular inorganic phosphate fraction and the easily hydrolyzable organic P compounds. In fertilized eggs, between 84 to 88 per cent of the P^{32} entering the eggs is found in the easily hydrolyzable fraction, indicating that the accumulation of phosphate by fertilized eggs involves primarily its incorporation in the easily hydrolyzable P compounds.

In cells actively metabolizing substrate the intracellular inorganic phosphate concentration may be markedly lower than in slowly metabolizing cells, devoid of or with a limited supply of substrate (yeast: MacFarlane, 1936, 1939; bacteria: Wiggert and Werkman, 1938, O'Kane and Umbreit, 1942; brain tissue: Schachner *et al.*, 1942; retinal tissue: Bumm and Fehrenbach, 1931; liver: Lundsgaard, 1938). Furthermore, many investigators have shown that orthophosphate rapidly enters and accumulates in actively metabolizing cells (diatoms: Ketchum, 1939a, 1939b; yeast: Hevesy *et al.*, 1937, Mullins, 1942; bacteria: Vogler and Umbreit, 1942, Wiggert and Werkman, 1938, O'Kane and Umbreit, 1942, Hotchkiss, 1946; brain tissues: Schachner *et al.*, 1942). When the same cells are devoid of substrate, phosphate ions enter slowly and the cells may even lose phosphate to the external medium.

Unfertilized sea urchin eggs, at least after a period of sojourn in sea water, present the picture of cells with limited available or utilizable substrate. They possess a characteristically low metabolic rate (Borei, 1948), have a high inorganic phosphate content, a relatively low content of easily hydrolyzable P (see also

Chambers and Mende, 1953b), may slowly lose phosphate to the external medium, and P penetrates the eggs at an extremely slow rate (Brooks and Chambers, 1954). However, after the eggs are fertilized, the eggs behave as if an abundant supply of substrate had been made available, or had become utilizable. The oxygen consumption increases, the inorganic phosphate content of the eggs is strikingly lowered, the quantity of easily hydrolyzable P increases (see also Chambers and Mende, 1953b), and the eggs now accumulate phosphate, absorbing it from the external medium.

The fertilized eggs would appear to accumulate orthophosphate against a concentration gradient of a thousand-fold or more (compare column 2, Tables II and III with column 3, Table IV). This, however, is unlikely since the analytically determined inorganic P content of cells probably represents, in addition to the true intracellular ionic orthophosphate, hydrolysis products of highly labile phosphate esters and orthophosphate which, in the living cell, had been present in undissociated salt-like complexes. The binding of orthophosphate by electrostatic forces has been shown to occur, for example, in the protein aldolase (Velick, 1949). Denaturation of proteins may abolish their ability to bind anions (Klotz and Urquhart, 1949). Furthermore, the anions of an extracting agent, such as trichloroacetic acid, would tend to displace phosphate ions which, in the living cells, had been present in undissociated salt-like complexes and in equilibrium with free orthophosphate ions. It is proposed that in the living sea urchin eggs the actual concentration of free ionic orthophosphate is only a fraction of the analytically determined inorganic P. Following fertilization of the eggs, along with the demonstrated decrease in concentration of the analytically determined inorganic P, the concentration of free orthophosphate ions in the egg protoplasm may be reduced to such a low order of magnitude as to favor the entry of orthophosphate from the external medium.

The hypothesis has been advanced that the penetration of orthophosphate into cells requires esterification at the cell surface. The marked effects of changes in temperature and of certain metabolic inhibitors (Kamen and Spiegelman, 1948, Villee *et al.*, 1949) on the rate of penetration of orthophosphate have been cited in support of this hypothesis. However, Jacobs and co-workers (1935) have emphasized that changes in temperature may cause marked shifts in "equilibrium" states, and such alterations would have to be taken into account before the effects of temperature changes on the rate of penetration of orthophosphate could be properly evaluated. Similarly, metabolic inhibitors must induce profound changes in "equilibrium" states within cells. For example, Spiegelman, Kamen and Sussman (1948) have shown that azide prevents the decrease in concentration of intracellular inorganic P which normally occurs when yeast ferments glucose.

The claim has also been made that orthophosphate must enter cells by a process of esterification at the cell surface, since the specific activity of the P in certain organic compounds may be higher than that of the intracellular inorganic P (*e.g.*, Lindberg, 1950). Such an interpretation from specific activity measurements is open to serious question, since the analytically determined inorganic P of cells is undoubtedly derived from several different components, and does not represent the true ionic orthophosphate content of the living cell.

The observed great differences in the rates of penetration of orthophosphate into cells at different levels of metabolic activity may just as well be explained by

changes in "driving forces" such as the rate at which orthophosphate is esterified within the cells, and changes in the concentration gradient of free orthophosphate ions.

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Summary

1. The concentration of phosphate in the external medium of a suspension of unfertilized *Strongylocentrotus* eggs remains constant, or increases, while in a suspension of fertilized eggs, the concentration of phosphate in the external medium decreases.

2. Fertilized *Strongylocentrotus* eggs absorb P^{32} and phosphate from sea water at identical rates, revealing that the exchange of phosphate between the cell interior and the external medium is inappreciable.

3. The rate at which phosphate is removed from sea water by fertilized *Strongylocentrotus* eggs is relatively independent of the external concentration as long as this exceeds 15 to 20 micrograms P per liter.

4. When unfertilized and fertilized sea urchin eggs are continuously exposed to sea water containing P^{32} and more than 20 micrograms P per liter, 95.9 to 96.4 per cent of the P^{32} which enters the eggs is found in the trichloroacetic acid-soluble fraction, with 3.6 to 4.1 per cent of the P^{32} being recovered in the acid-insoluble fraction. The distribution of P^{32} between these two fractions is not significantly different in the unfertilized, as compared to the fertilized eggs. Although a slightly lower proportion of P^{32} was found in the acid-insoluble residue of unfertilized eggs, outward leaching of P^{32} during the washing of the unfertilized eggs may well account for the difference noted.

5. If fertilized *Lytechinus pictus* eggs containing P^{32} are suspended in a non-radioactive medium shortly after insemination, the proportion of P^{32} in the acid-insoluble fraction increases from 3.6 per cent at the two-celled stage to 7.0 per cent at the blastula stage.

6. The concentration of inorganic P in the trichloroacetic acid-soluble extracts of the eggs decreases prominently following insemination. A corresponding increase occurs in the quantity of P liberated after 10 minutes' hydrolysis of the extracts in 1 N HCl at 100° C.

7. The major portion of the P^{32} which enters the eggs is found in the easily hydrolyzable P fraction of the trichloroacetic acid-soluble extracts. After fertilization, the proportion of P^{32} in the easily hydrolyzable P fraction increases.

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A STUDY OF THE MECHANISM INVOLVED IN SHIFTING OF THE PHASES OF THE ENDOGENOUS DAILY RHYTHM BY LIGHT STIMULI

FRANK A. BROWN, JR.,[1] MILTON FINGERMAN AND MARGARET N. HINES

Department of Biological Sciences, Northwestern University, and The Marine Biological Laboratory, Woods Hole, Mass.

A persistent diurnal rhythm of color change in the fiddler crab, Uca, was first reported by Abramowitz in 1937, and has been abundantly confirmed by numerous investigators since that time. The character of the chromatophore rhythm is such that the crabs darken by day and blanch by night owing to dispersion and concentration, respectively, of the melanin within their melanophores. This rhythm has been discussed most recently and described in considerable detail by Brown, Fingerman, Sandeen and Webb (1953). These investigators demonstrated that the rhythm not only persists for long periods in constant darkness, but actually increases in amplitude to reach a maximum value only after ten days to two weeks. This high value was then observed to persist without diminution for the longest period of observation which was about a month. Furthermore, there appeared to be no measurable drift of the rhythm away from its normal phase relations with solar day-night, indicating the mechanism to have a remarkable precision of frequency determination. This frequency was shown by Brown and Webb (1948) to be independent of temperature over the twenty degree range from 6 to 26° C.

Although the mechanism appears to be a moderately stable one in the fiddler crab, it was shown by Brown and Webb (1949), Webb (1950), and Brown, Fingerman, Sandeen and Webb (1953) to be capable of having its phases readily shifted by light-to-dark and dark-to-light changes under certain circumstances. Examples of these shifts are (1) a backward shift of 4 to 5 hours in the phases of the rhythm by three consecutive midnight-to-6 A.M. periods of illumination of animals otherwise in continuous darkness, (2) a forward shift of about 6 hours in animals whose rhythm had been inhibited by several days sojourn in continuous bright illumination and then were placed in constant darkness at 7 A.M., and (3) a shift of about twelve hours, or in other words a reversal, of phases by a few cycles of illumination from 7 P.M. to 7 A.M. and darkness from 7 A.M. to 7 P.M. Brown and Webb (1949) using illuminations of 150, 80, and 50 ft. c. found that the brighter the light the sooner the reversal. With the highest illumination, reversal occurred on the first day and with 40 ft. c. it occurred on the fourth. Once shifted, the rhythms are as stable in their new phase relations as they were in their original normal ones.

The experiments to be described were performed in order to gain further insight into the mechanism involved in inducing persistent shifts and certain other modifications in the endogenous diurnal rhythm.

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Materials and Methods

For the experiments to be described, 400 fiddler crabs were collected at Chapoquoit, near Woods Hole, Massachusetts at about three o'clock on the afternoon of June 24, 1952. They were kept in the natural daylight of the laboratory until 7:00 P.M. when they were divided into sixteen groups of 25 crabs each and placed in white enamelled pans in sea water to a depth of about a centimeter. One group, the control one, whose normal rhythm was to be determined was placed in darkness and left for the duration of the experiment. The remaining fifteen groups, the experimental ones, were placed in the conditions of illumination to which they were to be exposed during the night (7 P.M. to 7 A.M.) and the next morning placed in the lower illumination or darkness to which they were to be exposed by day (7 A.M. to 7 P.M.).

The illuminations for the experiments were obtained by frosted incandescent lamps of various wattages held at different distances above the white pans containing the animals. The illuminations were measured with a Weston photometer. The nighttime-daytime illuminations for the fifteen groups of animals subjected to the alternating illuminations were, in ft. candles, respectively: (1) 100–80, (2) 100–50, (3) 100–10, (4) 100–2, (5) 100–0, (6) 50–10, (7) 50–5, (8) 50–2, (9) 25–10, (10) 25–5, (11) 25–2, (12) 10–5, (13) 10–2, (14) 5–2, (15) 2–0.

The temperatures in the inside rooms of the Marine Biological Laboratory in which the experiments were carried on did not show any significant diurnal variation, and there was an irregular variation of only three or four degrees at most during the course of the eleven days in which the experiment was carried out. All the animals, furthermore, both experimentals and controls, were subjected to essentially the same temperature conditions other than the rhythmic differences in heat radiation during the periods of illumination.

The experimental groups were subjected to the twelve-hour alternating conditions of illumination for six days; at 7 P.M. on June 30, they were placed in constant darkness. Beginning at 11 P.M. and continuing at six-hour intervals (11 P.M., 5 A.M., 11 A.M., 5 P.M., . . .) for four daily cycles the average chromatophore stage of ten crabs randomly sampled from the fifteen experimental groups and the controls were staged by the method of Hogben and Slome (1931). Through the next or fifth daily cycle the chromatophores were staged at hourly intervals.

Experiments and Results

A summary of the results is found in Table I.

1. *Controls:* The control group possessed a high-amplitude rhythm at the time the staging of chromatophores commenced and showed no significant increase during the five-day period of study. The highest value was found at 11 A.M., and the lowest at 11 P.M. in every daily cycle.

2. *100–80 ft. c.:* In these, it is evident that there was a strong initial depression of rhythm-amplitude, which rapidly diminished during the five days. In every cycle the maximum stage was now at 5 A.M. and the minimum at 5 P.M.

3. *100–50 ft. c.:* This group, unlike the preceding, exhibited little or no initial amplitude depression nor increase during the period of observation. It resembled the preceding in having the maximum average stage always at 5 A.M. and the minimum at 5 P.M.

TABLE I

The average stage of melanin dispersion at each of four times of day, under constant conditions, for Uca which were earlier subjected to five days of higher illumination by night and lower illumination by day

Illum. (ft. c.)	11 P.M.	5 A.M.	11 A.M.	5 P.M.	Illum. (ft. c.)	11 P.M.	5 A.M.	11 A.M.	5 P.M.
Control	1.0†	2.3	5.0*	3.8	50–2	2.7*	1.0†	1.6	1.8
	1.0†	1.9	4.7*	4.1		2.8*	1.4	1.0†	1.6
	1.2†	1.9	4.5*	3.5		3.4*	1.3†	1.9	2.8
	1.1†	2.4	4.7*	4.4		3.0*	1.1†	1.9	2.4
	1.3†	3.1	4.5*	3.5		4.0*	1.4†	1.9	3.9
100–80	1.3	1.8*	1.3	1.0†	25–10	1.1†	1.9*	1.8	1.6
	1.2	1.2*	1.0	1.0†		1.0†	2.0*	1.7	1.3
	1.3	3.6*	2.9	1.1†		1.2†	3.2*	3.2	2.3
	1.9	4.3*	3.0	1.1†		1.6†	3.2	4.2*	1.7
	2.3	4.0*	2.3	1.0†		1.3†	3.7*	2.7	1.7
100–50	3.4	4.7*	2.2	1.2†	25–5	1.2†	1.3	2.2	2.4*
	2.4	4.2*	1.7	1.2†		2.3	2.1†	2.1	2.5*
	3.4	4.2*	3.5	1.1†		2.2†	2.9	3.9*	3.2
	3.0	4.5*	2.7	1.2†		2.4†	3.1	4.0	4.0*
	3.4	3.8*	3.1	1.4†		1.8†	2.9	2.8	3.5*
100–10	3.5*	3.0	1.6	1.0†	25–2	1.3	1.0†	1.2	1.3*
	1.8	3.0*	1.4	1.0†		1.3	1.0†	1.3	1.3*
	2.8	3.6*	2.7	1.2†		1.4	1.0†	2.0	2.6*
	2.5	3.1*	1.6	1.0†		2.6	1.0†	2.6	3.7*
	2.5	3.2*	1.7	1.1†		2.6	1.0†	1.3	2.9*
100–2	1.8*	1.3	1.0†	1.4	10–5	1.2	1.1†	1.5	2.0*
	1.7*	1.0	1.0†	1.0		1.3†	1.3	2.2*	1.8
	1.9	1.6†	2.0	2.7*		1.2†	2.0	3.4	3.6*
	2.7	2.3	2.1†	3.0*		1.7†	1.7	3.0	3.3*
	3.0	2.1	2.0†	3.2*		1.0†	2.4	3.0*	2.7
100–0	4.4*	3.8	1.0†	3.2	10–2	1.6	1.3†	1.9	2.1*
	3.9*	3.0	1.0†	1.9		1.0†	1.4	1.6*	1.4
	4.4*	1.7	1.0†	2.4		1.7†	2.1	2.3	2.9*
	3.8*	2.1	1.0†	2.5		2.4	1.9†	3.1*	2.7
	4.0*	2.7	1.0†	3.0		2.5	1.7†	1.7	3.0*
50–10	2.1	3.9*	2.4	1.2†	5–2	1.0†	1.2	2.1	2.3*
	2.1	4.4*	3.2	1.2†		1.1†	1.8	2.7*	2.1
	1.9	4.9*	4.7	1.5†		1.1†	1.5	3.0*	2.6
	1.5	4.3*	3.6	1.2†		1.0†	1.0	4.0*	2.2
	2.4	4.6*	3.6	1.5†		1.2†	1.6	2.6*	2.5
50–5	1.9*	1.8†	1.7	1.6	2–0	2.4	1.2†	2.8	2.9*
	1.6	1.4†	1.4	1.8*		2.0	1.3†	2.9	3.4*
	1.3†	1.8	2.6*	2.2		3.1	1.7†	3.7	4.0*
	2.3	1.8†	2.7*	2.6		3.2	1.4†	3.7	4.5*
	2.6	1.9†	3.0*	2.4		2.6	1.5†	2.5	3.4*

* Signifies maximum values for a cycle.
† Signifies minimum values for a cycle.

4. *100–10 ft. c.:* The group subjected to these illuminations appeared to show an intermediate degree of depression in amplitude from the beginning and no systematic increase thereafter; but still again, the maximum was nearly always at 5 A.M. and the minimum always at 5 P.M.

5. *100–2 ft. c.:* This group showed initial amplitude depression with a rapid increase during the five days in darkness. Now, the maximum evidently was between 5 and 11 P.M. and the minimum between 5 and 11 A.M.

6. *100–0 ft. c.:* This was the only one of the experimental groups which had undergone a complete reversal of phases. The amplitude was very great from the start and showed no increase. The maximum pigment dispersion was seen at 11 P.M. and the minimum at 11 A.M. in every cycle.

7. *50–10 ft. c.:* This group showed little or no initial depression of amplitude. The maximum occurred at 5 A.M. and the minimum at 5 P.M. in every instance.

8. *50–5 ft. c.:* This group initially showed not only great depression in rhythm amplitude, but initially almost an absence of a recognizable daily cycle. A clear daily cycle did reappear in two or three days and gain in amplitude. But now, strangely, the maximum was close to or at 11 A.M. and the minimum at 5 A.M.

9. *50–2 ft. c.:* This group also showed an initial low amplitude of rhythm, but one which increased rapidly. The maximum value was at 11 P.M. in every cycle and the minimum at 5 A.M. in all but one.

10. *25–10 ft. c.:* An initial amplitude depression was observed in this group but it rapidly disappeared. The time of maximum dispersion appeared to lie between 5 and 11 A.M. and the minimum was always at 11 P.M.

11. *25–5 ft. c.:* The rhythm of this group exhibited an initial depression, and the maximum was between 11 A.M. and 5 P.M. with the minimum at 11 P.M. in four out of the five cycles.

12. *25–2 ft. c.:* In this group, there was an initial depression in amplitude which rapidly vanished; the daily cycle was reasonably symmetrical with an unequivocal maximum at 5 P.M. and minimum at 5 A.M.

13. *10–5 ft. c.:* A great reduction in rhythm amplitude was initially seen in this group. The time of maximum pigment dispersion appeared clearly to lie between 11 A.M. and 5 P.M., and the minimum close to 11 P.M.

14. *10–2 ft. c.:* Again, the amplitude gradually increased during the five days in darkness. The time of the maximum was sometimes seen at 11 A.M. and sometimes at 5 P.M. The minimum, on the other hand was distributed between 11 P.M. and 5 A.M. during the five daily cycles.

15. *5–2 ft. c.:* The amplitude for this group was rather low throughout the five days. The minimum was invariably at 11 P.M. and the maximum nearly always at 11 A.M.

16. *2–0 ft. c.:* There was only slight, rapidly transitory amplitude depression in this group. The maximum value was clearly at 5 P.M. and the minimum at 5 A.M.

Discussion and Conclusions

Viewing the data of Table I as a whole, it is clearly evident that even though the animals have all received in common a higher illumination during 12 hours from 7 P.M. to 7 A.M. and a lower one from 7 A.M. to 7 P.M., there is to be found among the results a whole spectrum of apparent kinds and degrees of shifts of the phases

of the daily rhythm. Furthermore, for any given illumination combination, although the amplitude of the rhythm might undergo considerable change during the five-day period of observation after the animals were left in darkness, the phases of the rhythm in no case showed any evidence of a drift in one direction or the other. The forms of the daily variations of Table I were essentially confirmed on the last day of the five-day series when staging of chromatophores was performed hourly.

In order to obtain a better estimate of the direction and amount of shift in the times of the phases of the rhythms in the experimental crabs away from the con-

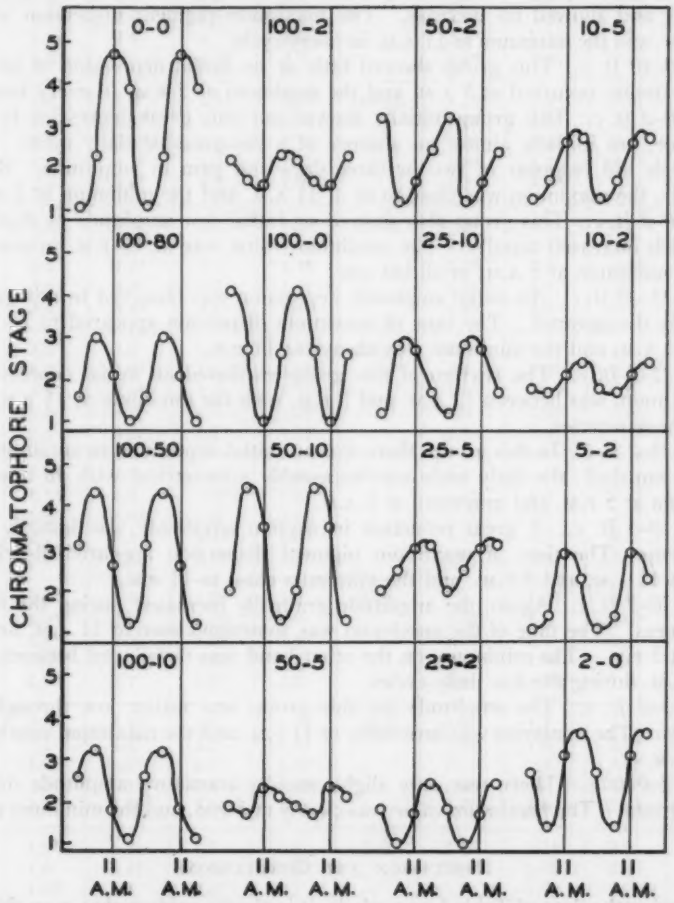

FIGURE 1. The average form and phase relations of the daily melanophore rhythm of *Uca pugnax* in constant darkness after five days of subjection to various higher illuminations by night and lower ones by day. The night-day illumination combinations are indicated for each rhythm.

dition in the controls, it was considered a reasonable procedure to average the values for each time of day for the five days for each group, plot these averages against time of day, and draw smooth curves. This has been done, and two average daily cycles are illustrated in Figure 1. Obviously, the amplitudes illustrated are only the mean ones for the five-day periods, but the forms of the curves and the relationship of the times of their various phases to the actual hour of the day are the factors of chief concern in this consideration. These two factors showed no significant alteration during the five days as is quite evident from Table I.

An examination of Figure 1 clearly shows that the control curve obtained in continued darkness can be illustrated as a more or less sinusoidal one with a

TABLE II

The number of hours by which the phases of the persistent daily rhythm are shifted forward (+) or backward (−) by alternating periods of brighter illumination by night and dimmer illumination by day

Shift of maximum (hrs.)	Illumination night-day (ft. c.)	Shift of minimum (hrs.)	Illumination night-day (ft. c.)
+12	100–0 50–2	+12	100–0
+9	100–2	+9	100–2 50–2
+6	25–2 10–2 2–0	+6	25–2 10–2 2–0
+3	25–5 10–5	+3	50–5
0	50–5 5–2	0	25–5 10–5 5–2
−3	25–10	−3	25–10
−6	100–80 100–50 100–10 50–10	−6	100–80 100–50 100–10 50–10

maximum at 11 A.M. and a minimum at 11 P.M. In sharp contrast with this control, those crabs which had been subjected to 100 ft. c. by night and darkness by day, though similarly capable of depiction as a relatively simple sinusoidal rhythm, were in almost exactly opposite phase.

Although the great majority of the experimental groups appear capable of description in terms of simply a change in amplitude of the cycles, together with more or less displacement forwards or backwards in time relative to the control, there are a few that appear quite definitely to have undergone a modification of form capable of approximate description in terms of the times of maximum and minimum having

been displaced to different extents away from the controls. This is evident in the 50–2 ft. c. group in which the minimum appears to have been displaced to the right by only about 9 hours while the maximum was being shifted by 12 hours.

Comparable differential shifts are also apparent in the 25–5 ft. c. group, where the minimum appears unshifted while the maximum is moved about three hours to the right, in the 10–5 ft. c. group where almost exactly the same situation obtains, and in the 50–5 ft. c. group where the maximum is probably unshifted and the minimum moved to the right, by about three hours.

The differential shifts just described give rise to persistently skewed daily cycles as is evident from all of these curves.

If one considers the 100–0 ft. c. group with a completely reversed rhythm as having both maximum and minimum displaced to the greatest extent, and this to be 12 hours to the right, or forward, in each case, all the other experimental groups tend to fall naturally into a series of lesser amounts of shift to the right, through no shift, and finally to a maximum amount of shift to the left, or backward, of 6 hours. This graded series is described in Table II.

Study of Table II reveals that with 100 ft. c. by night and darkness by day, both maximum and minimum points in the daily cycle are considered as shifted 12 hours forward. For the same illumination by night, an increase in the illumination by day progressively decreases the amount of the shift. With 2 ft. c. by day, the shift is only about 9 hours; with 10 ft. c. by day, the shift is 6 hours backwards, and this value is not exceeded for 50 and 80 ft. c. by day. Similarly for 50 ft. c. by night the greatest amount of shift, 9 to 12 hours forward, occurs when the day value is 2 ft. c. At 5 ft. c. by day the amount of shift has dropped to 0 to 3 hours; and elevating the day to 10 ft. c. produces again the maximum shift backwards of 6 hours.

A comparable series is seen for 25 ft. c. by night. Two ft. c. by day yields a 6-hour shift forwards, 5 ft. c. a 0- to 3-hour shift forwards, and 10 ft. c. a 3-hour shift in the opposite direction. Similarly for 10 ft. c. at night, 2 ft. c. by day gives a 6-hour forward shift, while 5 ft. c. by day yields only a 0-3-hour one.

Five ft. c. by night and two by day produces no change in either direction. Two ft. c. by night and darkness by day gives a 6-hour shift forward.

It is evident that 25 ft. c. at night is not sufficiently great to produce the maximum shift of 6 hours backwards, only 3 being possible. And 10 ft. c. by night appears to be capable of producing no backward shift whatsoever.

These results suggest that for darkness by day, there is a direct relationship between the number of hours of shift and the illumination by night. One-hundred ft. c. gave the maximum shift of 12 hours forwards; two ft. c. gave only about 6 hours forwards. It seems reasonable to postulate that for other values between 100 ft. c. and darkness, other degrees of shift ranging down to no shift at all might be found.

In summary, the amount and direction of shift of the phases of the persistent diurnal rhythm appear to be determined in these experiments by at least two factors. One is the strength of the stimulus in the form of a light increase at 7 P.M., and the other is the intensity of the illumination during the period from 7 P.M. to 7 A.M. A minimum intensity of 50 to 100 ft. c. during the 7 P.M. to 7 A.M. period is necessary to produce the maximum 6-hour shift backwards, and the minimum strength of the stimulus of illumination change at 7 P.M. necessary to produce the

total 12-hour forward shift is produced by some light change between 0–100 or 0–50 ft. c. on the one hand and 2–100 or 2–50 ft. c. on the other.

All of these results, and others that have been obtained in previous work with respect to shift in phases of the diurnal rhythm by illumination changes, are capable of being explained in terms of one hypothesis which will now be presented.

Let it be assumed that the endogenous rhythm in those crabs is one in which the general form of some key aspect of the rhythm can be described as illustrated in Figure 2. Instead of being composed of symmetrical cycles, it is skewed so that one limb is of about 6 hours in duration and the other one about 18 hours. Let the normal relationship of the phases of this endogenous rhythm to the solar day be

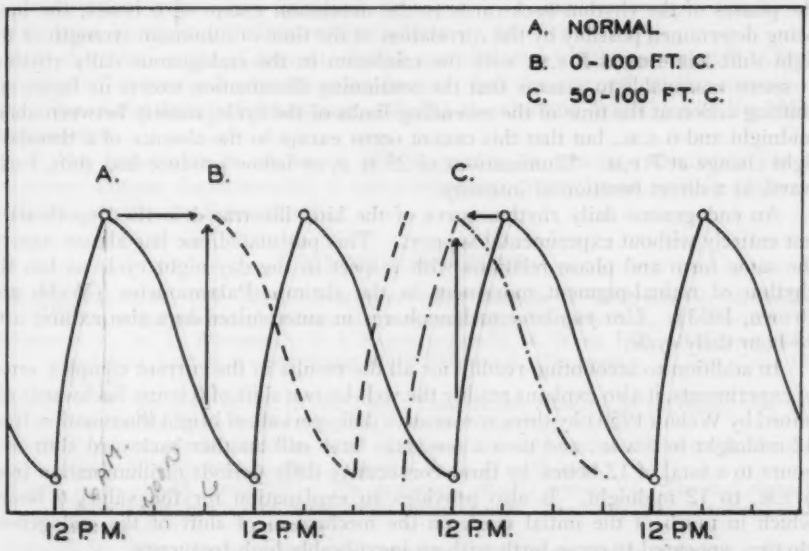

FIGURE 2. Diagram describing an hypothesis for the mechanism of inducing persistent shifts in rhythm phase by light stimuli. Solid curve—normal phase relations. Broken curve—rhythm reversed by a strong illumination change stimulus at 7 P.M. Dot-dash curve—6-hour backward shift by bright illumination from 6 P.M. to 6 A.M. (See text for discussion.)

as indicated in the figure, with the minimum occurring at about midnight and the maximum at about 6 A.M. Since in nature this maximum is normally correlated with the rapid morning increase in illumination and the succeeding 12 hours of the rhythm is normally correlated with the period of daytime, let it be assumed that both of these are normally involved in the bringing of the phases of the endogenous rhythm into their usual and characteristic relationship to the daily light cycle.

In this hypothesis, a strong stimulus in the form of a large increase in illumination at 7 P.M. (*e.g.*, 0 to 100 ft. c. . . .) induces the endogenous state normally correlated with the maximum in the endogenous cycle, or what would amount to a displacement of the phases of the rhythm 12 hours to the right or forward. Smaller

increases in illumination at 7 P.M. (*e.g.*, 2 to 100, 2 to 25, and 5 to 10 ft. c.) would have progressively less effect and the cycle of the endogenous rhythm would be displaced progressively less to the right and only to a degree that the displaced cycle at 7 P.M. was brought into an equilibrium for the light-increase stimulus. To an intensity change represented by the 5 to 50 or 2 to 5 ft. c. shifts at 7 P.M., the endogenous rhythmic mechanism appears normally to be in equilibrium. At still lower strengths of the "shift stimulus," *e.g.*, 10–25 and 10–50, and 10–100 ft. c., the strength can be considered less than the equilibrium one, but now the phases of the rhythm will not automatically shift backwards. The backward shift, if permitted by the intensity-change stimulus strength, is induced by the continuing illumination. A value higher than 25 ft. c. during the 7 P.M. to 7 A.M. period is necessary to move the phases of the rhythm backwards to the maximum extent of 6 hours, the limit being determined possibly by the correlation of the time of minimum strength of the light-shift stimulus at 7 P.M. with the minimum in the endogenous daily rhythm. It seems reasonable to assume that the continuing illumination exerts its backward shifting action at the time of the ascending limbs of the cycle, namely between about midnight and 6 A.M., but that this cannot occur except in the absence of a threshold light change at 7 P.M. Illuminations of 25 ft. c. or below produce less shift, backward, as a direct function of intensity.

An endogenous daily rhythm curve of the kind illustrated in the hypothesis is not entirely without experimental support. This postulated one has almost exactly the same form and phase relations with respect to the day-night cycle as has the rhythm of retinal-pigment movement in the shrimp, Palaemonetes (Webb and Brown, 1953). *Uca pugilator* melanophores in autotomized legs also exhibit a 6-18-hour daily cycle.

In addition to accounting readily for all the results in the current complex series of experiments, it also explains readily the well-known shift of 6 hours backwards obtained by Webb (1950) by three consecutive daily periods of bright illumination from 12 midnight to 6 A.M., and then a few days later still another backward shift of 6 hours to a total of 12 hours, by three consecutive daily periods of illumination from 6 P.M. to 12 midnight. It also provides an explanation for the value, 6 hours, which in much of the initial work on the mechanism of shift of the endogenous rhythm, appeared to come forth with an inexplicably high frequency.

Speculating further upon the actual nature of the physiological processes involved in these light-induced shifts probably would not be very productive at this time. One of numerous possibilities could be that the hypothetical curve describing the diurnal rhythm is a curve describing the intensity of a physiological state which may be altered by light stimuli. A change from darkness to light at any time during the endogenous reduction of this state could elevate it in proportion to the strength of the stimulus. Once abruptly altered in this manner, the endogenous, temperature-independent mechanism could take over with the cycle exhibiting a renewed start at a point in the cycle normally characterized by this higher level. Increase in level of this rhythmic state would be the equivalent, during the descending portion of the curve, to moving the phases of the cycle to the right. On the other hand, the ascending limb of the curve describing an increase in the intensity of the state of the rhythm could be capable of being accelerated by light up to the degree that is nearly instantaneous, provided the phases of the rhythm had not been rigidly de-

termined at an earlier stage in the same cycle by threshold change from light to darkness for that particular phase of the cycle. This would amount to a shift of the phases of the cycle to a maximum of 6 hours to the left. This would not shift further to the left, because the presence of a sub-threshold dark-to-light stimulus for 7 P.M. would earlier in each cycle have freed the cycle to move to a point with the minimum at 7 P.M.

Summary

1. A study was made of the mechanism of reversal of phases of the persistent daily rhythm in the fiddler crab, *Uca pugnax*, by illumination by night and darkness by day.

2. Fiddler crabs were subjected to a series of combinations of brighter illumination by night and dimmer illumination by day.

3. A graded series of amount of shift was obtained which was capable of being interpreted in terms of two operating factors: (a) the strength of the stimulus in the form of the dark to light change, and (b) the absolute brightness of the higher illumination.

4. An hypothesis is advanced which appears to account adequately for all currently known characteristics of the mechanism of persistent shift in phases of the daily rhythm by light stimuli.

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THE DISTRIBUTION OF PHOSPHORUS (P^{31} AND P^{32}) IN DORSAL AND VENTRAL HALVES OF THE RANA PIPIENS GASTRULA [1]

PHILIP GRANT [2, 3]

Department of Zoology, Columbia University, New York 27, N. Y.

Studies of the distribution of enzymatic activity and oxygen consumption in the amphibian gastrula have led to hypotheses concerning the role of metabolic gradients in development (Child, 1941). Although considerable confusion exists as to whether such gradients have been established, the appearance of the dorsal lip does delineate a region of distinct morphogenetic activity from one presumably less active (the ventral half), and does suggest that a comparison of their respective metabolic activities would reflect these morphogenetic differences.

Considering the important biological and metabolic role of phosphate compounds, it was felt that a study of their distribution between these two morphogenetically distinct regions would reveal specific metabolic differences of a more convincing nature. Furthermore, radioactive phosphorus was used to make possible an analysis of shifts of phosphorus, either from one region to another, or from one component to another.

Methods

Rana pipiens females were weighed and injected with pituitary glands to induce ovulation. They were then injected intraperitoneally with approximately 0.1 mc. of P^{32} in the form of H_3PO_4. Forty-eight hours later, the eggs were harvested and fertilized. They were allowed to develop in large finger bowls at 15° C. until Shumway stage 10.

The jelly and vitelline membrane were removed and the gastrulae were then dissected into two halves as shown in Figure 1. Dorsal and ventral halves were collected in separate stender dishes standing in an ice water bath. From twenty to forty halves were transferred to 12-ml. graduated centrifuge tubes and washed twice with full strength Holtfreter's solution. All operations were carried out in full strength Holtfreter's solution in vessels kept in ice water and the homogenization and extraction were completed in a 4° C. cold room.

The fractionation procedure was a modified Schmidt- Thannhauser extraction (1945). The fractions isolated were the following: (1) total acid-soluble phosphorus, (2) "desoxyribonucleic acid phosphorus," (3) "ribonucleic acid phosphorus," (4) "phosphoprotein phosphorus," (5) "phospholipid phosphorus," and (6) residue phosphorus.

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[2] Postdoctoral Public Health Research Fellow, National Cancer Institute.

[3] Present address: Institute for Cancer Research, Philadelphia 11, Pa.

After alkali digestion of the defatted material, the supernatant was poured off, DNA was precipitated by the addition of an HCl-TCA mixture and DNA phosphorus determined according to the procedure outlined by Sze (1953). The residue remaining in the alkali digest was analyzed as "residue phosphorus."

After precipitation of DNA, the remaining supernatant was precipitated with magnesia mixture overnight to obtain the inorganic phosphorus liberated from phosphoprotein. The resulting filtrate was hydrolyzed in 60% perchloric acid and analyzed as "ribonucleic acid phosphorus."

All fractions isolated were hydrolyzed in 60% perchloric acid in a sand bath until clear, and inorganic phosphorus was precipitated as the magnesium ammonium complex with magnesia mixture. The precipitates were collected on filter paper and mounted on brass discs for counting, which was done with a Geiger-Muller end window tube (3.3 mg./cm.^{2}) using a Nucleonic RC 2 scaler. All samples were corrected for decay and the instrument was checked daily against a standard beta source.

FIGURE 1. Dissection of gastrula. V = ventral half; D = dorsal half.

The precipitates were eluted in 1 N sulphuric acid and the phosphorus determined by the method of Berenblum and Chain (1938) using the vessel described by Wiame (1947).

Six separate experiments were completed, each run in duplicate, with appropriate reagent blanks.

The fractions isolated represent heterogeneous groups of phosphate compounds with a wide range of different origins (Grant, 1953). Furthermore, there is considerable doubt as to the extent of purity of these isolated fractions, particularly those fractions that may be contaminated with inorganic phosphorus (Davidson *et al.*, 1951). Because of the relative nature of the data, however, it was assumed that any significant differences between dorsal and ventral halves should be evident using this technique.

RESULTS AND DISCUSSION

In Table I, the distribution of phosphorus (P^{31} and P^{32}) in dorsal and ventral halves of the gastrula is shown, in absolute values and in percentages of total phosphorus and total radioactivity. No significant differences are evident in any of the fractions. In two experiments, whole gastrulae were extracted along with

the halves to determine the efficiency of recovery, which was fairly good. The low recoveries of acid-soluble phosphorus and phospholipid phosphorus may be attributed to loss of blastocoel fluid in the case of the former and loss of yolk granules during the dissection procedure in the case of the latter. The data do illustrate that dorsal and ventral halves have a similar distribution of phosphorus and that cleavage produces a uniform apportionment of the egg constituents.

In a recent study of the regional chemical differences in the frog gastrula (Barth and Sze, 1953), gradients of lipid and of total nitrogen (animal-vegetal) were demonstrated; however, no dorso-ventral gradient was evident, which agrees with the absence of a phosphorus gradient shown in Table I. Although the analyses of Barth and Sze were performed on several small regions of the gastrula, their data, calculated on the basis of dorsal and ventral regions (to make them approximately equivalent to half-gastrulae analyzed here), exhibit no dorso-ventral differences.

TABLE I

Distribution of phosphorus (P^{31} and P^{32}) in whole gastrulae and in dorsal and ventral halves

Fraction	Whole			Dorsal half			Ventral half		
	μgms. P^{31}	% total P^{31}	% total P^{32}	μgms. P^{31}	% total P^{31}	% total P^{32}	μgms. P^{31}	% total P^{31}	% total P^{32}
Acid-soluble P	77.0	5.35	90.00	32.5	5.10	86.74	30.5	4.34	87.58
Ribonucleic acid P	45.0	3.18	1.03	20.0	2.82	2.82	25.5	3.65	2.33
DNA P	5.0	0.39	1.34	2.5	0.40	1.23	2.0	0.33	1.14
Phospholipid P	283.5	19.65	4.78	112.8	17.47	5.79	127.7	18.12	6.09
Phosphoprotein P	974.5	67.55	2.41	489.5	73.68	4.02	517.3	72.81	3.20
Residue P	58.0	3.95	0.18	15.0	3.46	0.75	23.5	4.38	0.97

Values for μgms. P^{31} expressed as micrograms of phosphorus per 100 embryos or per 100 half-embryos. Values for % total P^{32} obtained from values expressed as counts per minute per whole or half-embryo.

The values for all fractions, except DNA phosphorus and RNA phosphorus, compare closely with Kutsky's (1950) results. The low values for these latter two fractions may have been due to a failure to obtain complete precipitation with magnesia mixture, since the amounts involved are relatively small. In addition, loss of RNA phosphorus may have been due to adsorption onto the magnesium ammonium precipitate of phosphoprotein phosphorus. However, the relative values are significant and these indicate that no differences exist.

The per cent distribution of P^{32} also illustrates that no significant differences are apparent when the halves are compared to each other, or to the whole embryo.

When the number of cells in the two regions is considered (Sze, unpublished data), Table II is the result. Since the ventral half contains fewer, larger cells (approximately 15,800 cells with an average volume of 67,500 μ^3 compared to the dorsal half with 18,100 cells with an average volume of 52,600 μ^3) the results are to be expected. The larger ventral cells contain a greater proportion of cellular constituents, particularly yolk granules, which represent about 70% of total egg phosphorus (Grant, 1953). Thus, phosphoprotein phosphorus and phospholipid

TABLE II

Distribution of phosphorus (P^{31}) per cell of half gastrulae

Fraction	Dorsal	Ventral
Acid-soluble P	0.178	0.193
Ribonucleic acid P	0.109	0.161
Desoxyribonucleic acid P	0.014	0.013
Phospholipid P	6.155	8.075
Phosphoprotein P	26.750	32.750
Residue P	0.082	0.149

Values expressed as micrograms P^{31} per cell $\times 10^5$.

phosphorus, the major constituents of yolk phosphorus (Panijel, 1950), exhibit the greatest differences.

The specific activity data (Table III) suggest that differences between dorsal and ventral halves may exist. However, these differences are insignificant when tested by the comparison of individuals method. The high specific activity of the acid-soluble fraction in the whole embryo may be attributable to the retention of blastocoel fluid, possibly rich in highly active inorganic phosphorus.

It is possible that differences could be made more evident (that is, if they exist) if smaller regions of the gastrula were compared; regions similar to those analyzed by Barth and Sze (1953). The dissection into half gastrulae includes large areas of tissue of similar metabolic activity such that small differences between halves are masked. Possibly, in later stages of gastrulation, where metabolic differences are more pronounced (Brachet, 1950), dorsal and ventral halves would exhibit divergencies in their phosphorus distribution and specific activity.

The residue phosphorus exhibited activities of the same order of magnitude as the ribonucleic acid fraction, suggesting that it might be undigested nucleic acid. It is also possible that the residue might be metaphosphate as described by Wiame (1947) in yeast. The possible existence of metaphosphate is interesting in the light of recent findings by Berg (unpublished data) that there is a strong metaphosphatase present in the developing embryo.

No specific activity values are reported for DNA phosphorus since those experiments which yielded activities for this fraction failed to yield detectable amounts of phosphorus.

TABLE III

Relative specific activity $\times 10^5$.
Whole and half gastrulae

Fraction	Whole	Dorsal	Ventral
Acid-soluble P	202.25	142.48	141.72
Ribonucleic acid P	4.15	7.16	4.39
Desoxyribonucleic acid P	——	——	——
Phospholipid P	2.98	2.78	2.38
Phosphoprotein P	0.38	0.43	0.31
Residue P	0.58	3.43	5.09

Values expressed as $\frac{\text{cts./min./}\mu\text{gms. P}}{\text{act. injected/wt. gms.}}$.

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Kutsky (1950) reports significant shifts in activity from gastrulation to neurulation. These have also been found by the author (unpublished data). This points to the need for continued study of more advanced stages of gastrulation to demonstrate and localize these changes. This report represents the completed portion of such experiments now in progress.

Summary

1. A preliminary investigation of the distribution of phosphorus in dorsal and ventral halves of the *Rana pipiens* gastrula was undertaken to demonstrate the possible existence of a metabolic gradient of phosphate compounds correlated with the apparent morphological gradient. Radioactive phosphorus was employed to permit an analysis of shifts of phosphorus.

2. A modified Schmidt-Thannhauser extraction procedure was applied to dorsal and ventral halves of stage 10 (Shumway) embryos obtained from a frog injected with approximately 0.1 mc. of P^{32} before inducing ovulation. Total acid-soluble phosphorus, RNA phosphorus, DNA phosphorus, phosphoprotein phosphorus, phospholipid phosphorus and a residue phosphorus were extracted and analyzed for specific activity.

3. The distribution of phosphorus (P^{31} and P^{32}), expressed either as micrograms P^{31} per half embryo or as per cent of total P^{31} or P^{32}, exhibited no significant difference between dorsal and ventral halves. However, expressed as micrograms P^{31} per cell, a ventral-dorsal gradient was apparent. Data expressed as specific activity (counts per minute per microgram P^{31}) exhibit no significant differences.

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THE RESPIRATION OF NORMAL LARVAE OF TEREDO BARTSCHI CLAPP[1]

CHARLES E. LANE, J. Q. TIERNEY[2] AND R. E. HENNACY

The Marine Laboratory, University of Miami, Coral Gables, Florida

The free-swimming, infective larval stage of *Teredo* in local waters does not significantly exceed seventy-two hours in duration (Isham and Tierney, 1953). During this time the animals have not been observed to feed (Lane, Posner and Greenfield, 1952). The pre-attachment activities of the animal must be presumed to be powered chiefly by glycogen. This is deposited in the ovum in granular form during oogenesis. Additional glycogen may be contributed to the larva during the time that it is actually embedded in the maternal gill. At the termination of this transient, free-swimming stage, the larvae attach themselves permanently to a wooden substratum within which they spend the rest of their adult life span. A cellulase enzyme system exists in both larval and adult *Teredo* (Greenfield and Lane, 1953). This enzyme complex may significantly facilitate the invasion of wood by the larvae.

The act of penetration of wood renders the larva virtually immune to environmental hazard except for substances in solution either in the wood itself or in the water which constitutes the respiratory stream. Thus it is that preventive measures, to be effective, must be directed against the larva during the vulnerable first seventy-two hours of its free-living life.

A sensitive index of physiological condition, or of the effectiveness of sub-lethal concentrations of toxic substances, is provided by the rate of oxygen consumption of living systems. Some of the parameters of normal respiration in free-living, pre-attachment stages of *Teredo* were delimited preliminary to a study of the effectiveness of some toxic agents. Details of this latter phase of the investigation will be presented elsewhere. It is the purpose of the present communication briefly to describe the methods and some of the results observed in the study of normal animals.

Materials and Methods

All larvae employed in this study were reared in the laboratory by methods described by Lasker and Lane (1953).

Oxygen consumption was measured in a capillary microrespirometer (Fig. 1). It consists of a pear-shaped chamber blown in one end of capillary tubing. The volume of the chamber varied in different respirometers over the range of six to 125 microliters. The volume should be kept as small as possible to increase the stability of the system (Tobias, 1943). The opposite end of the capillary tubing

[1] Contribution No. 120 from the Marine Laboratory, University of Miami. These studies were aided by a contract between the Office of Naval Research and the University of Miami in cooperation with the U. S. Navy Bureau of Yards and Docks.

[2] Present address: Hydrographic Office, U. S. Navy, Washington, D. C.

bears an inside syringe-taper ground joint. This seats in the outer matching ground joint of the thermobarometer or compensation chamber. This latter portion of the apparatus should be as large as is consistent with ease of manipulation. We have generally sought to have its volume at least 1000 times that of the respirometer chamber. This insures maximum sensitivity of the system. The upper end of the compensation chamber is closed by a stopcock. In use the entire assembly is immersed in a constant temperature water bath maintained at 25.0° C.

The chamber is first loaded with a single animal confined in a droplet of medium whose volume varied for different respirometers between one and ten microliters. This volume provides a mass of medium from 100 to 1000 times the volume of the organism. The isolation of the larva and the determination of the volume of the medium can be effected most easily by making use of specially drawn and calibrated micropipettes. These may be actuated either by a syringe device or by a mouthpiece similar to that of a hemocytometer pipette. Calibration of pipettes and other micro-glassware is readily accomplished with the micrometer burette described by

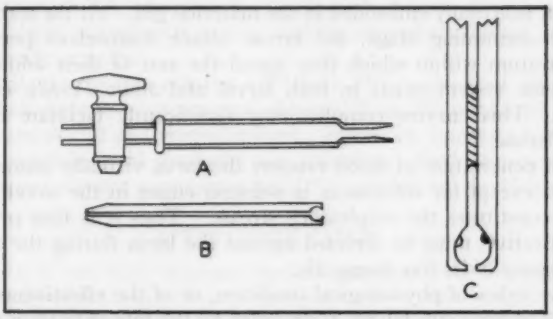

FIGURE 1. Sketch of components of the capillary microrespirometer. A. Thermobarometer with outer syringe-taper ground joint in end opposite stopcock. B. Microrespirometer with matching inner syringe-taper ground joint in end opposite respirometer bulb. C. Enlarged view of respirometer bulb to show the disposition of droplets and the kerosene indicator fluid.

Scholander (1942). Various loading pipettes may be calibrated to deliver precisely known total volumes. The delivered volume, of course, will include the volume of the organism. Separate pipettes are constructed for each respirometer, and are then used only with that particular apparatus.

The droplet of medium and larva is delivered onto one wall of the respirometer chamber. The chamber wall is previously rendered hydrophobic by the application of a suitable silicone coating. Under these conditions the integrity of the droplet of medium is retained for long periods of time. It has, for example, frequently been possible to make continuous observations of the oxygen consumption of a single larva during periods as long as twenty-four hours without opening the sealed system.

After the respirometer has been charged with the animal and medium, a droplet containing one to five microliters of alkali, either 10% NaOH or 5% $Ba(OH)_2$, is placed on the contralateral wall. The indicator fluid in the capillary is kerosene which has been distilled at 250° C. after exhaustive oxidation with concentrated sulfuric acid for several days. The open end of the respirometer is sealed with a

non-oxidizing wax. For best adhesion and complete sealing it is preferable to employ a wax of comparatively low melting point. With the upper stopcock of the compensation chamber open, the two portions of the apparatus are united, seated and the joint is sealed with the same wax which was used to close the lower end of the respirometer. The assembly is then placed in the water bath and permitted to come to temperature equilibrium. Under our conditions a steady state is generally reached by respirometers without respiring tissue within thirty minutes.

With the system sealed and equilibrated the mode of operation is as follows. The larva extracts dissolved oxygen from the sea water medium. This creates a diffusion gradient across the air-water interface, as a consequence of which additional oxygen diffuses into the water from the air phase. The loss of oxygen from the air phase causes a decrease in pressure in the air phase which is reflected in displacement of the kerosene meniscus. The position of the meniscus is observed with a compound microscope equipped with a long-focus objective. It is clear that

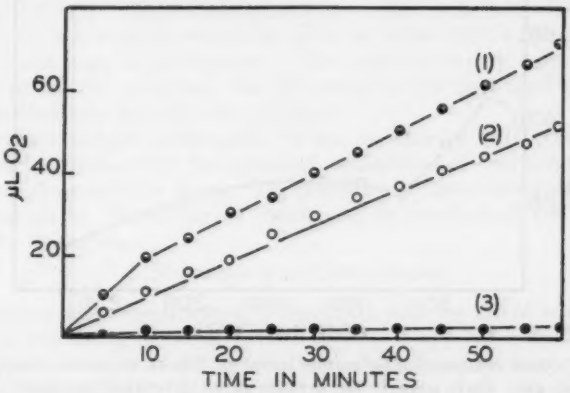

FIGURE 2. Average rate of oxygen uptake by 24-hour larvae of *Teredo*. Each point is the average of from three to 17 determinations on different animals. (1) in the presence of *M*/1000 glucose in sea water, (2) normal sea water and (3) blank.

the sensitivity of the apparatus is limited by the resolving power of the optical system. In our studies we have found it convenient to detect a displacement of ten microns. This represents a change in volume of 0.002 mm.[3] In the terminology proposed by Scholander and Evans (1947) this is 2.0 γl. Naturally the sensitivity of the entire system could be increased either by increasing the magnification of the optical system or by decreasing the diameter of the capillary out of which the respirometer is constructed. For the present study sufficient sensitivity was provided by the dimensions described.

RESULTS

In Figure 2 are shown average oxygen consumption values for over one hundred individual determinations. The lowest curve is the sea water blank. Inasmuch as this generally consisted simply of the water in which the Teredids were living at the time of their capture, it usually contained microorganisms which showed a small

but significant oxygen consumption. Blank figures were always subtracted from the oxygen consumption of experimental animals. The middle curve shows the average rate and magnitude of oxygen consumption by normal 24-hour larvae. The upper curve shows the oxygen consumption by normal 24-hour larvae when the sea water medium had been rendered 0.001 *M* by the addition of appropriate amounts of glucose.

Figure 3 shows the change in rate of oxygen consumption with increasing age of the larvae. The increase during the first twenty-four hours is real and significant. From seventy-two hours to three hundred hours the decrease in oxygen consumption is progressive. This decline is associated with general involutional changes in the larva which will lead to its death by three hundred hours if it is denied access to wood.

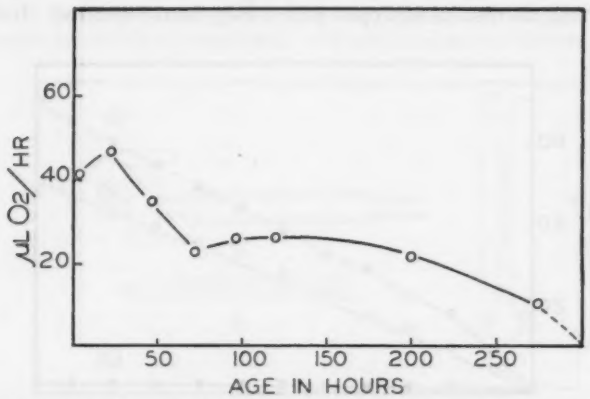

FIGURE 3. Oxygen consumption by normal larvae of *Teredo* at various times after release from the maternal gill. Each point is the average of all determinations made for that age. Data on 38 different larvae are presented in the curve.

DISCUSSION

These data serve to emphasize both similarities and differences between the larval behavior of *Teredo bartschi* Clapp and *T. navalis*. Imai, Hatanaka and Sato (1950) have described the large-scale culture of larvae of *T. navalis*. They emphasize the importance of suitable supplies of food organisms in the maintenance of normal growth of the cultures. They also figure the larvae at various stages of its free-living life. In *T. navalis* this pre-attachment period may occupy as much as 34 days. During this time the larva continues to grow. The maximum pre-attachment size is approximately 245 microns in diameter.

T. bartschi, on the other hand, has been shown to be 250 microns in diameter when it is released from the maternal gill. The length of its normal free-swimming life does not exceed four days.

In a previous publication from this laboratory (Lane, Posner and Greenfield, 1952) the statement was made that larvae of *T. bartschi* "do not appear to feed." It was thought that the large supplies of glycogen which are characteristic of the

mature oocyte were sufficient to power the pre-attachment activities of the larvae during a short free-living life. There can now be little doubt that this conception is erroneous in view of the observed oxygen consumption of the larva during this portion of its life. Elementary calorimetric considerations show that larvae of *T. bartschi*, which weigh approximately 10 micrograms alive, and contain close to 60% moisture, fall far short of containing sufficient metabolic fuel materials to justify the measured oxygen consumption during their free-living existence. The deficit must be made up by the ingestion of microorganisms from their environment.

The large glycogen stores probably represent emergency reserves which are used after 72 hours. At this stage the larvae cease to swim and assume a pedestrial mode of progression. Clearly they do not come into contact with the same number of suspended food organisms when crawling in two dimensions as when swimming relatively rapidly through three dimensions.

The increased oxygen consumption at twenty-four hours is correlated with behavioral changes in the free-swimming larvae which have been described by Isham and Tierney (1953). These investigators have shown that crawling with the aid of the muscular foot replaces swimming with the velar cilia as the chief method of locomotion at this stage of development. The enhanced oxygen consumption during this portion of the life cycle may also be related to the post-natal development of enzymatic mechanisms for complete glycolysis.

The increased oxygen consumption in the presence of $M/1000$ glucose can be most reasonably attributed to the increased metabolism of microorganisms present in the medium along with the larva. The possibility of direct absorption of dissolved organic materials by *Teredo* larvae should not be overlooked, but it is certainly not proved by this work.

Summary and Conclusions

1. A capillary microrespirometer is described, with the aid of which the normal respiration of free-living larvae of *Teredo bartschi* Clapp has been studied.

2. Oxygen consumption during the total three hundred-hour pre-attachment life averages approximately 25 microliters per hour, when measured at 25° C. At twenty-four hours there is a significant augmentation. After seventy-two hours the rate of oxygen consumption decreases regularly until the death of the larva. The significance of these alterations in rate is discussed. Differences between larval behavior of *T. bartschi* and *T. navalis* are described.

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NUTRITION OF THE SEA URCHIN, STRONGYLOCENTROTUS PURPURATUS[1]

REUBEN LASKER AND ARTHUR C. GIESE

Hopkins Marine Station of Stanford University, Pacific Grove, California

The gut of the purple sea urchin, *Strongylocentrotus purpuratus,* reveals a mass of algae in various stages of decomposition. Algae contain relatively small amounts of nutrients which are readily handled by enzymes ordinarily present in animals, but they possess galactans, alginic acid, agar and possibly some cellulose, none of which are digested by man or most animals. The urchins in the course of evolution may have developed enzymes which have enabled them to use these materials, or, like many of the ungulate herbivores, they might harbor bacteria, or, like the termites, they might shelter protozoans which perform this role for them.

The first study was therefore concerned with the role of the digestive enzymes present in the gut of the urchin. The second consisted of studies of the digestive action of the flora of the urchin gut. The third was concerned with the over-all nutritional economy of the sea urchin.

Materials and Methods

Sea urchins were collected on the Monterey Peninsula at Yankee Point below Carmel Highlands and at Pescadero Point. A large number of urchins were planted just outside the Hopkins Marine Station to make them available for experiments requiring an occasional fresh specimen. While some of those transplanted died or disappeared, a fairly large number took hold and fed upon the prevalent coralline algae. Monthly studies were made on the urchins from Pescadero Point, because they could always be obtained even in rough weather, whereas those at Yankee Point sometimes became inaccessible in stormy weather. All the locations from which the urchins were taken were free of industrial waste and relatively free of sewage. The sea urchins brought in fresh monthly were kept in the laboratory in aerated rapidly running sea water. Even so, most of them aggregated near the top of the water in a tank, except when they wandered in search of food. Unless only a relatively small number of urchins were kept in an aquarium, they became unhealthy in time.

For determination of the sugar and nitrogen content in the body fluid, ten urchins were sacrificed the first day and ten the second after collection. The pH of body fluid was determined with a Beckman pH meter directly in the field, and

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the pH of gut contents as soon as possible after opening a normal, healthy urchin in the laboratory.

The body fluid was withdrawn from an urchin by excising Aristotle's lantern and pouring out the contents of the coelom. The fluid was allowed to stand and the clot, containing wandering blood cells, was removed by filtration. Reducing sugars were determined in the filtrate by the method of Somogyi (1945, 1952). The non-protein nitrogen (NPN) in the coagulum-free filtrate of body fluid was determined by the standard Kjeldahl procedure, the proteins being first precipitated with trichloracetic acid (TCA).

Experimental

1. Structure of the digestive tract of the sea urchin

The mouth opening between the teeth of Aristotle's lantern leads through an oesophagus to the stomach-intestine which is suspended in the coelom by a mesentery. The oesophagus possesses well developed villus-like papillae which contain glands, perhaps producing mucus. The intestine has two turns; looking in at the mouth of the urchin the first turn is clockwise and the second, doubling back upon this, is counter-clockwise. The intestine is also lined by a glandular epithelium in which secretory cells are found, some forming glands possessing a body and a neck which opens into the digestive cavity. The connective tissue of this portion of the gut is inconspicuous and the epithelium appears to be bounded by the visceral peritoneum. Since the wall of the gut is so thin it is probable that absorption can occur readily through any part of it.

2. Feeding habits of the sea urchin

Over the last ten years, sea urchins kept in aquaria have been fed a great variety of foods. If starved, they were found to ingest almost anything offered them such as boiled eggs, boiled potatoes and vegetables, as well as fresh vegetables, but not leaves of geranium or *Pelargonium*. However, meat and fruits were taken in preference to vegetables. In nature the sea urchin feeds upon various algae (green, red and brown) as well as upon the "surf-grass," *Phyllospadix*. In local areas the diet may be largely restricted to the most abundant alga. In the laboratory, the sea urchins were usually fed the red alga, *Iridophycus flaccidum*, because of its availability and its acceptability to the urchin.

The ingested food apparently remains in the gut for a long period of time since during starvation in an aquarium, feces were ejected for two weeks, suggesting a very slow rate of digestion. When urchins brought in from the field were roughly handled, they eliminated considerable amounts of material for a short period. When slowly eliminated, the algae in the feces were found to be fairly completely decomposed and were heavily laden with bacteria. If feeding was continuous, so was defecation, and the algal pellets appeared to be less completely digested.

When an urchin which had been starved for some time in the laboratory was given food, it quickly fed to capacity with any material, algal or otherwise, upon which tests were desired. Starvation for two weeks was used as standard practice since in this time the gut will have become considerably, if not completely, cleared of contents.

Considering the low temperature of the water in which the sea urchins live, the

rate of digestion is not surprising. The water temperature for Moss Beach varied from 9.2 to 15.5 and at Stillwater Cove from 8.7 to 16.1° C. during the year. On the rare occasions when the urchins were exposed to sunlight at low tide the temperature may have risen considerably over this.

3. Digestive enzymes of the intestine

Since the normal food of the purple sea urchin consists of algae, the digestive enzymes most likely to be found in the gut are those which can handle the nutrients found in the algae. The protoplasm of algal cells, of course, contains protein, and floridean starch is stored in red algae. However, much organic material is present in red algae in the form of galactans or galactans mixed with other materials, *e.g.*, agar and various gums. Enzymes which can handle proteins (proteases), starch (amylase) and the various substances peculiar to algae are therefore of special interest. Tests were made for each of these.

For extraction of enzymes, the gut contents were flushed out with sterile 3% NaCl, and the gut rinsed three times in the salt solution. The entire digestive tracts of several animals were then ground with crushed Pyrex glass in a mortar with a small amount of water, extracted with buffer solution and centrifuged. The buffer used in extraction was a 0.5 *M* McIlvaine buffer of pH 6.8–7.0. This pH was used because the first measurements of the pH of a sea urchin gut gave readings of 6.8–7.0. Subsequent measurements indicate that a pH of 7.2 to 7.3 is probably more nearly correct for a freshly opened gut. When the urchin is kept in the laboratory for a brief time the pH falls.

That a protease is present was easily demonstrated by mixing the gut extracts with casein, adding toluol to inhibit bacterial growth and determining the increase in NPN with lapse of time. Since some NPN appears in the control without casein, the data given in Figure 1 are for the differences between the two. The data demonstrate that the NPN rises rapidly after action of the enzymes on the protein.

That an amylase is present was shown by the appearance of reducing sugar in a sample of boiled starch mixed with gut extract (toluol as antiseptic). As seen in Figure 1 considerable reducing sugar appears after action of the enzymes in the extract on the boiled starch. As a control the gut extract was incubated without starch and tested for appearance of reducing sugar.

Agar and other gums contained in the algae are made up principally of polymerized galactose often combined with other substances. The ability of the sea urchin to digest agar was tested by adding the enzymes extracted from the gut to warm agar (about 37–40° C.) and mixing. The results, shown in Figure 1, may be considered negative since the very small change in reducing sugar concentration following exposure to the extracts is probably within the limits of error of the experimental method. The results for iridophycin, a galactan isolated from *Iridophycus flaccidum* by Hassid (1933), indicate that an iridophycase is present (Fig. 1). In all cases a control was incubated without the substrate and tested for reducing sugar. It would be desirable to test other substances peculiar to algae but they were not available in pure form for the tests.

4. Fauna and flora of the sea urchin gut

Ciliate protozoans occur in small numbers in the sea urchin gut, about ten to a hundred being counted per ml. of gut contents of several sea urchins. Several

species of *Entorhipidium* and *Lechriopyla mystax* have been identified (Lynch, 1929a, 1929b). Few were ever seen in division by Lynch; therefore they seem to represent a static population. The protozoans appear to ingest bacteria and small particles of well-disintegrated algae. While the activities of the protozoans, some of which live for several days outside the intestine, should be studied, it is unlikely that the protozoans contribute to the digestion of the algae because of their small numbers and their feeding habits.

Bacteria are present in the gut of the sea urchin in sufficient numbers to be of consequence. Almost every pellet of algal material in the second section of the intestine was found to be surrounded by a translucent membrane which, upon

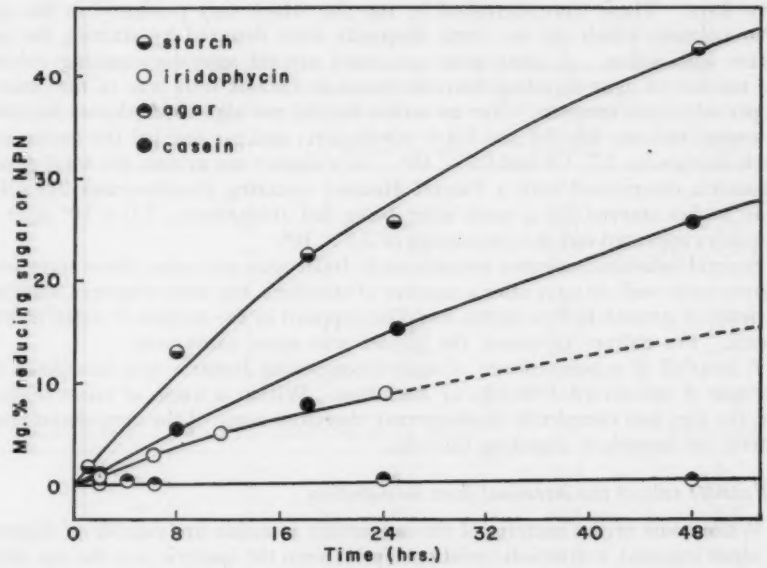

FIGURE 1. Rate of digestion of 1% casein by the extract of the intestine of the purple sea urchin at pH 6.8 and 30° C., and of 1% boiled starch, 0.1% agar and 0.02% iridophycin by the extract of the intestine of the purple sea urchin at pH 6.8 and 30° C.

microscopical examination, proved to be a film lined with coccus and rod-shaped bacteria. The films develop soon after the algal pellets enter the gut. When defecated the enveloped pieces were largely digested and no longer recognizable as algal pellets. An unenveloped piece of alga may become colorless but the cell walls remain intact and while no bacteria are seen within the walls, they occur around the cells damaged by the teeth. The envelope of mucilaginous material develops in the gut of the sea urchin, but not in cultures of the bacteria from the sea urchin gut growing on algae *in vitro*.

Since the enzymes from the sea urchin gut do not digest intact algal tissue, yet the latter disintegrates in the gut, it would appear that either the algae autolyze or

the bacteria digest them. Experiments eliminated the first possibility: algae in sea water under toluene did not autolyze. Attempts were therefore made to determine whether the bacteria from a sea urchin were capable of digesting the algae. An autoclaved sample of the red alga *Iridophycus* was inoculated with a loopfull of bacteria removed aseptically from the hind gut of a sea urchin. In one week at room temperature the algae had completely decomposed. The experiment was repeated several times.

Next, to test whether the bacteria in the lumen of the gut digest agar, a constituent of the cell walls of many algae, a sample of gut fluid was aseptically removed and transferred onto a 2% agar-sea water plate. The sample was poured over the plate or spread over it with a sterile glass spreader. Many colonies appeared within a few days. These were identified by the pits which they produced in the agar. Other colonies which did not form deep pits were detected by staining the agar surface with iodine. A clear area was noted around agar-decomposing colonies. The number of agar-digesting bacteria found in various tests was of the order of 10^6 per ml. of gut contents. For an urchin fed the red alga, *Iridophycus flaccidum*, the counts indicate 1.8, 2.1 and 1.4×10^6 bacteria and for one fed the brown alga, *Alaria marginata*, 1.7, 1.8 and 0.7×10^6. In a similar sea urchin, the total number of bacteria determined with a Petroff Hausser counting chamber was 2.6×10^{11}. In an urchin starved for a week after being fed *Iridophycus*, 1.0×10^6 agar decomposers appeared out of a population of 3.3×10^8.

Several individual colonies transferred to fresh agar-sea water plates were found to grow quite well on agar after a number of transfers, but some required additional nutrients or growth factors which could be supplied in the extract of algae or yeast extract. For culture purposes, the former was more convenient.

A loopfull of a pure culture of agar-decomposing bacteria was inoculated into a sample of autoclaved *Iridophycus flaccidum*. Within a week, at room temperature, the alga had completely disintegrated, therefore some of the agar-decomposing bacteria are capable of digesting this alga.

5. Possible role of the intestinal flora in nutrition

While some of the bacteria of the sea urchin intestine are capable of digesting the algae ingested, a symbiotic relationship between the bacteria and the sea urchin is not thereby proven. It is possible that the sea urchin maintains itself on the more readily available nutrients in the algae which it is capable of digesting—*i.e.*, the floridean starch and the proteins of the algae. The presence of enzymes capable of digestion of starch and protein supports this possibility.

On the other hand, the intestinal flora may render the nutrients in the cells of the algae more readily available to the sea urchin, by digesting the cell walls. However, it seems unlikely that the bacteria would spare the more generally utilizable nutrients such as starch and protein while selectively digesting the generally less available cell wall materials. It seems more likely that the enzymes present in the foregut of the sea urchin digest the more readily available materials in the algal cells before the bacteria have multiplied sufficiently to offer competition, and that the residue is then attacked by the bacteria which gradually decompose the algal cell walls.

The bacteria nevertheless may contribute to the host by digesting the structural

components of the algae and releasing some of the nutrients which can then be absorbed by the host into the body fluid. This would constitute a type of symbiosis. Sugar is mobilized (Table I) in the body fluid of a sea urchin soon after a meal of algae but this could be explained adequately as a result of digestion of starch in the algae by the amylases of the gut. Reducing sugars did not accumulate in cultures of the agar-digesting bacteria tested. It is possible that they are present only transiently in the intestine and might be absorbed to a small extent. Cultures of bacteria tend to become acidified and fatty acids may be demonstrated in the culture fluid and in the body fluid of the sea urchin. Identification tests indicate lactic acid, judging from the position of the spot in chromatographic analysis (Reid and Lederer, 1952) and the Friedemann-Graeser determination (1933).

TABLE I

Reducing sugar in the body fluid of the sea urchin after starvation for two weeks and refeeding or injection of glucose

Fed	Days fed	Weight of food ingested in grams	Wet weight of urchin	mg. % reducing sugar
Iridophycus (red alga)	0	0	37.5	0
	1	1.33	42.4	12.0
	2	2.07	57.1	4.5
	4	3.43	40.5	1.3
	8	6.86	41.9	1.0
Boiled potato	0	0	36.9	0
	1	2.89	47.8	47.0
	2	4.69	48.4	62.0
	4	4.55	38.5	1.7
	8	10.09	43.9	24.3
Glucose injected	Hours time lapse	Mg. glucose injected		% glucose withdrawn
	0	0.0	34.5	0
	1	1.0	54.2	64.5
	2	1.0	41.4	78.0
	4	1.0	39.0	95.8
	8	1.0	37.9	88.5

The nature of the relationship between the sea urchin and the bacteria could be ascertained if the bacterial flora of the intestine could be removed and replaced at will. Killing the flora by a meal of $CuSO_4$, as has been done with some vertebrates, kills the sea urchin as well. Defloration by high oxygen tensions is not practical since the bacteria present in the gut are only facultative anaerobes, not obligate like the termite protozoa. Raising sea urchin larvae aseptically to the adult stage is not likely to be successful with present techniques. A mixture of streptomycin and penicillin, 50 ppm in gelatin, was unsuccessful in killing the bacteria in the gut of the urchins tested. Therefore a crucial direct test for digestion in the absence of bacteria cannot be performed. Only circumstantial evidence can therefore be adduced at present, to support the possibility of symbiosis between the sea urchin and the bacteria, but it is not so overwhelming as to exclude other possibilities.

6. Reducing sugar in the body fluid and glycogen stores in tissues

Reducing sugar is usually present in the body fluid of the sea urchin as shown in Figure 2. The amount of sugar in individual cases varies from 0 to 13 mg. per cent. The sugar practically disappears after starvation (Table I). However, it is mobilized quickly in such an animal after a meal of algae or boiled potato (Table I). In the latter case the sugar rose from 0 to 62 mg. per cent in two days, the highest value ever obtained with this species of sea urchin over the course of a year of analyses. The intestinal amylases may be responsible for digestion but since bacteria are present in the gut their possible share in the digestion of the starch cannot be ignored.

Glucose injected into a starved sea urchin rapidly disappears from circulation (Table I). This suggests that the tissues take up glucose and other reducing

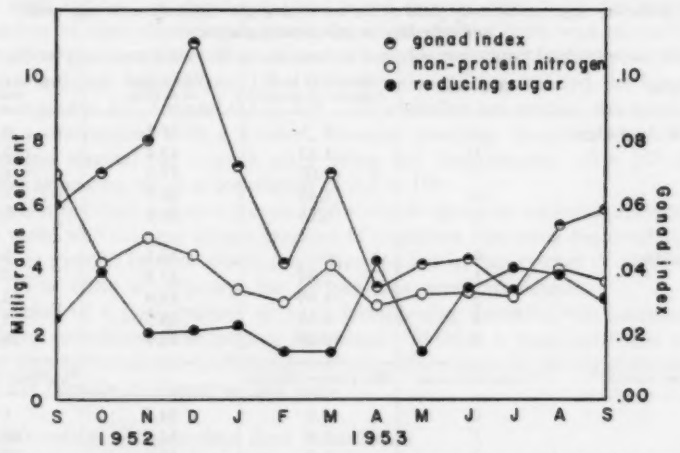

FIGURE 2. The gonad index (volume of gonad divided by the wet weight of the sea urchin) correlated with the average content of non-protein nitrogen (NPN) and reducing sugar in the body fluid of the purple sea urchin obtained monthly by analyses of twenty urchins.

sugars and store them in some insoluble form. Preliminary experiments have demonstrated glycogen in various tissues, but most appears in the intestine (Hilts and Giese, 1949). To determine the storage of glycogen a male sea urchin was drained of body fluid and the gut tissues were rinsed free of contents in distilled water and dried in an oven. The pulverized material, including the test, was extracted with alkali and the glycogen precipitated in alcohol. The glycogen present in a sample of the sea urchin was found by the method of Meyer (1943) to be 472 mg.% on the basis of dry weight, while 123 mg.% nitrogen was present. The amount of glycogen per unit weight of protoplasm was estimated as follows. Assuming that the protein of protoplasm contains all the nitrogen of the animal and that protein constitutes about 15% of the protoplasm, 3.84 units of glycogen are present per unit nitrogen or 0.62 unit of glycogen per unit protein, or almost 0.1 per cent glycogen in the protoplasm (muscle contains 1 per cent). The data

show that a considerable store of glycogen exists in the tissues and apparently the tissues draw upon this store for their respiratory activities. Stott (1931) found that in *Echinus esculentus* glycogen accumulated during growth of the gonads but declined just before the annual spawning in spring.

The avidity with which glucose is taken up following injection after starvation (Table I) suggests the possibility that the tissues of an urchin are starved for sugar. If this were true one might expect that addition of sugar would increase the respiration of excised tissues. Respiration of sea urchin intestine determined with the standard Warburg technique shows that this is not so. Regardless of whether glucose or yeast extract or both were added, the respiration of tissues freshly removed from a well-fed or a starved sea urchin and suspended either in sea water

TABLE II

Non-protein nitrogen (NPN) of the body fluid of the sea urchin after two weeks starvation and refeeding

Fed	Days fed	Weight of food ingested in grams	Wet weight of sea urchin	mg. % NPN
Iridophycus	0	0	48.0	1.20
	1	2.73	56.6	4.30
	2	2.87	51.2	0.80
	4	3.28	45.9	3.75
Boiled egg albumin	0	0	28.9	3.19
	0	0	31.0	3.20
	1	3.03	35.3	6.67
	2	4.72	45.0	8.20
	3	2.54	44.3	3.75

or in the body fluid of the sea urchin did not increase significantly over the endogenous value. The average Q_{O_2} of digestive tissue was found to be about 0.7 ml./mg./hr.

7. Non-protein nitrogen in the body fluid

Non-protein nitrogen (NPN) is generally present in the body fluid of a sea urchin to the extent of about 5 mg. per cent. Data (averages) for monthly samples of groups of 20 urchins tested for a year are given in Figure 2. The NPN does not decrease markedly after starvation (Table II), but increases markedly after feeding with a high protein diet (Table II) rising in a day to twice the value for a control, and to an even higher concentration the second day. It falls off again on the third day.

The NPN seems to be regulated to some degree, since unlike the reducing sugar, it never falls to zero even after prolonged starvation. Nitrogen compounds stored in tissues must be liberated upon starvation to maintain the supply of soluble NPN. Storage of the nitrogenous compounds in the gonad is suggested, since the gonad of a sea urchin starved for a month is almost completely resorbed.

An attempt was made to determine the nature of the NPN by paper chromatography. The filtered body fluid from which proteins were removed with TCA

was treated with Duolite C3 resin in the acid cycle. The anions were washed out with distilled water and the amino acids were then separated from the cations by removal with NH_4OH and chromatographed in butyl alcohol (Redfield, 1953; Slotta and Primosigh, 1951). Only spots for alanine and glutamic acid were located. If other amino acids are present they occur in amounts too small to be detectable by even the very sensitive method used.

8. Nutrition and the reproductive cycle

Collections of this species of sea urchins over many years clearly suggest cyclic gonadal activity. Sperm are available practically all year but eggs are available only during a limited portion of the year. However, the gonads of both males and females undergo cyclic variation in volume. The cycle is probably different for each ecological habitat since differences in maturity were observed between the development of gonads of urchins at Yankee Point and at Pescadero Point. To ascertain the nature of the cycles, the gonads were removed monthly from twenty animals and their volume was determined by immersion in a graduate partially filled with sea water. The volume of gonadal tissues, divided by the weight of the urchin, designated the *gonad index*, was used for comparison of data which are given in Figures 2 and 3.

The growth of the gonads and the development of large numbers of gametes mean a synthesis involving the conversion of a considerable amount of nutrient to protein and nucleic acid. The amount of nitrogen in a gonad is large—a ripe testis containing 2.32% and a ripe ovary, 3.95% nitrogen per unit dry weight; therefore the nitrogen present in an animal probably increase several-fold during the breeding season. However the monthly determinations of the reducing sugar and the NPN in the body fluid given in Figure 2 provide no evidence of excessive mobilization or withdrawal of NPN or glucose during the breeding season.

Discussion

Van der Heyde (1922) states that whereas the European sea urchin, *Echinus esculentus* is herbivorous and feeds largely on *Laminaria* and other algae, Neapolitan sea urchins such as *Sphaerechinus* and *Toxopneustes* may be carnivorous and may even capture various crustaceans. *Arbacia punctulata* appears to be omnivorous in nature since algae, brittle stars, hydroids and spicules of sponges are found in its gut (van der Heyde, 1922). *Strongylocentrotus droebachiensis* has similar feeding habits (Scott, 1901; Weese, 1926). The western purple sea urchin, *Strongylocentrotus purpuratus*, studied here was found to be quite omnivorous under laboratory conditions, but specimens found in the field invariably were feeding on any algae available or on the "surf-grass," *Phyllospadix*. The gut contents of hundreds of individuals examined appeared to consist entirely of algal pellets, although small amounts of animal food might have been missed since critical examination for this purpose was not attempted. *Lytechinus anamesus* also appears to be an algal feeder, judging from its depredations on algal beds used for commercial extracts (A. P. Steiner, personal communication).

Algae contain proteins and other constituents of protoplasm and may possess small amounts of sugar and starch. In place of the latter may be found sugar alcohols such as mannitol, dulcitol and sorbitol. However, the greater part of

the bulk of dry algae consists of the constituents of the cell walls and supporting materials. These are made up mainly of polysaccharides other than cellulose although the latter is sometimes found, *e.g.*, in some of the brown algae. Algin, found in the brown algae, is a condensation product of mannuronic acid, $(C_6H_8O_8)n$. Hassid (1936) has demonstrated sulfuric acid esters of galactans which make up to 40% of the dry weight of the red alga, *Iridophycus flaccidum*. When the sulfuric acid is split off, hydrolysis gives rise to pure galactose. Red algae also store floridean starch. Other compounds have also been demonstrated in algae but the knowledge of many is incomplete and the characterizations quite vague (see Blinks, 1951 for a review). Such lack of knowledge seriously hampers experiments on digestion of the algae by the sea urchin since it is generally necessary to use the entire algae rather than individual compounds.

Starch, glycogen, sucrose and maltose were found to be digested by ground-up intestine and contents of the sea urchin, *Echinus esculentus* (Roaf, 1908) and sucrose and protein by a similar preparation of *Strongylocentrotus droebachiensis* (Weese, 1926) and starch but not fats by *Sphaerechinus granularis* (Cohnheim, 1901; Scott, 1901). Weese (1926) failed to demonstrate lypolytic activity in *Echinus* but van der Heyde (1922) found evidence for it in *Arbacia*, the gut extracts of which developed acid when incubated with olive oil.

In the studies on *S. purpuratus* performed here, strong protease and amylase activity were demonstrated in ground, washed intestinal tissue, but no evidence was obtained for an invertase nor unequivocal evidence for an agar-decomposing enzyme, even though agar is ingested by hungry urchins. The sugar content of the body fluid of the starved sea urchin is not increased by a meal of agar. No increases in reducing sugar were observed nor were the algae decomposed in two weeks when they were mixed with the gut extract of the sea urchin and kept under pentachlorophenol. However, in one series of experiments in which the galactan iridophycin was mixed with the extract of the intestinal wall, an increase of reducing sugar was obtained, demonstrating the presence of an iridophycase in the gut. This experiment probably has more validity than those with algae, since in the latter case side reactions may occur by which the reducing sugar is bound. Other algal polysaccharides should be tested in a like manner, but none were available in pure form.

The possibility that bacteria might play a role in digestion has not been previously seriously considered although Weese (1926) observed bacteria present in a film about the algal particles in the gut of *S. droebachiensis*. The present study on *S. purpuratus* indicates that large numbers of bacteria are present, of which a considerable number are capable of digesting algae. The population of bacteria is even greater per unit volume than the figures given because for these determinations a 0.1-ml. quantity of gut contents was ground up with sand and an aliquot was counted or plated. The bacteria actually develop in films about the algal particles; therefore they occupy only a fraction of the volume sampled. The bacteria decomposing agar and other algal cell-wall materials might well be present in sufficient numbers to digest the algae in the gut of the urchin. Furthermore the food is retained in the intestine for a week to two weeks, a period of time adequate for even slow digestion.

It was not possible to determine whether the sea urchins could survive loss of their bacterial flora; therefore the role of the bacteria could not be defined. They

may be commensals which use what is left by the digestive enzymes of the sea urchin or they may be symbionts. The tentative conclusion is drawn that the sea urchin possesses enzymes which hydrolyze protein and starches of algae and perhaps slowly decompose some of the more resistant algal polysaccharides. In the latter action they may be greatly aided by the bacteria present in the gut.

Under normal nutritive conditions reducing sugar was almost always found in the body fluid of sea urchins taken in the field or feeding in the laboratory, the amount being greatest just after active feeding and least after starvation. Lang and MacLeod (1920) also report that little reducing sugar is present in the body fluid of the echinoderms which they tested, although Myers (1920) reported an unusually large content in *S. franciscanus*. The possibility exists that additional sugar is present in a combination with proteins or other nutrients as in some other invertebrates (Morel and Bellion, 1910).

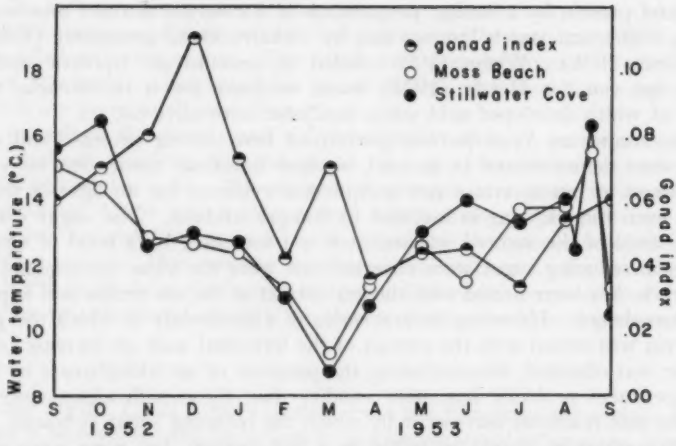

FIGURE 3. The gonad index (volume of gonad divided by the wet weight of the sea urchin) correlated with the water temperature during the year at Moss Beach, a habitat chosen because it lies on the open ocean, and at Stillwater Cover, a habitat chosen because it is sheltered.

The non-protein nitrogen (NPN) showed about the same variations throughout the entire year as the sugar but it was more closely regulated than reducing sugar during starvation. On starvation the NPN in the body fluid remained almost constant but the gonads shrank to a fraction of their former size. It would seem that the gonadal nitrogen was mobilized to maintain the NPN in the body fluid during starvation.

The gonads vary in size during the year and instead of a single growth period several were found in the monthly examinations made over a period of a year. The most striking was in December, but peaks occurred in March and June, presumably followed by spawning although all the spawning periods were not revealed by the monthly examinations. The gonadal cycle does not vary with temperature (Fig. 3) but may be related to cyclic changes in the algae providing

more food or more nutritious food at one time than at another. For lack of pertinent data in the literature it is impossible to test such a possible correlation, but on completion of a current study of algal populations in this region during the year, the necessary data may be available.

Summary

1. The intestinal tract of the sea urchin, *Strongylocentrotus purpuratus*, consists of two loops, the first clockwise, the second counter-clockwise (as seen looking in at the mouth). Both loops are well supplied with glands.

2. Experiments present evidence for an amylase and a proteinase in the sea urchin intestine but none for enzymes capable of digesting entire algae or agar. However, the extract of ground gut was capable of digesting iridophycin, a galactan from the red alga, *Iridophycus*.

3. Agar-digesting bacteria are present in the second loop of the intestine in large numbers from 10^6 to 10^7 per ml. of gut contents. Total bacteria as determined by a count with a Petroff-Hauser counting chamber reach the value of 2×10^{11} per ml. They are largely confined to the pellicle which surrounds each particle of alga.

4. The bacteria inoculated from the gut of the sea urchin are capable of completely digesting the alga, *Iridophycus flaccidum*, in the course of a week. Many of the intestinal bacteria are capable of digesting agar. Pure cultures of agar-decomposing bacteria were isolated from the intestine.

5. The isolated bacteria grown on algae or agar do not liberate reducing sugars into the culture medium.

6. While it seems likely that the sea urchin obtains some nutrient from the activities of its intestinal flora, proof for this is lacking.

7. Some glycogen is stored in the tissue of the sea urchin and the body fluid normally contains a small amount of reducing sugar. No striking changes occur in the latter during the breeding season, but the sugar falls to zero or nearly zero on starvation. Glucose injected into the body fluid is removed by the tissues.

8. The body fluid of the sea urchin always contains a small amount of non-protein nitrogen (NPN), even after two weeks of starvation, and no striking change occurs during the breeding season.

9. The stimulus that sets off the increased effectiveness of the economy of the sea urchin resulting in the growth of the gonads and accumulation of nitrogenous compounds in them is unknown. After gonads reach their maximal size spawning occurs.

10. Several cycles of gonadal growth and spawning are suggested but the data were not collected at sufficiently frequent intervals to make this certain.

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OXIDATIVE ENZYMES IN THE THORACIC MUSCLES OF THE WOODROACH LEUCOPHAEA MADERAE [1]

W. H. McSHAN, SOL KRAMER AND VERA SCHLEGEL [2]

Department of Zoology, University of Wisconsin, Madison, Wisconsin

Our present knowledge of cellular metabolism is based largely on results obtained from studies using mammalian tissues. The mechanisms of many of the enzyme-catalyzed reactions of respiration and glycolysis have been elucidated during the past twenty years. During recent years the methods developed for the study of mammalian tissues have been used to study some of the oxidative enzymes in insect tissues, with the result that considerable information has accumulated concerning certain of the oxidative enzymes in various insect tissues.

In this connection Barron and Tahmisian (1948) found that the oxygen consumption of muscle from male cockroaches, *Periplaneta americana,* is double that from female roaches. Sacktor and Bodenstein (1952) reported on the cytochrome oxidase activity of various tissues of the American cockroach, and Harvey and Beck (1953) studied in considerable detail the succinoxidase and cytochrome oxidase systems in the leg muscle of this form. They found that the succinoxidase activity of muscle from the male cockroach is three times that of muscle from the female. Spirtes (1951) demonstrated the presence of Krebs cycle enzymes such as aconitase, isocitric, malic and succinic dehydrogenases, fumarase and condensing enzyme, and also cytochrome oxidase and lactic dehydrogenase in the tissues of *Drosophila melanogaster;* and Bodenstein and Sacktor (1952) studied the cytochrome oxidase during metamorphosis of *Drosophila virilis;* Sacktor (1951a, 1951b, 1952) reported on the cytochrome oxidase activity of normal and DDT resistant house flies, *Musca domestica;* Sanborn and Williams (1950) studied the cytochrome system in the tissues of the Cecropia silkworm; Watanabe and Williams (1951) showed that succinic, α-glycerophosphate, malic and pyruvic dehydrogenases and cytochrome oxidase are present in the sarcosomes of insect muscles; and Collias, McShan and Lilly (1952) reported results of studies on the succinoxidase and cytochrome oxidase systems in the tissues of the large milkweed bug, *Oncopeltus fasciatus.* Bodine, Lu and West (1952) found marked differences in the succinoxidase activity in mitotically active and blocked cells of the developing embryo of the grasshopper, *Melanoplus differentialis.*

Investigations of this kind serve to clarify further our knowledge of the relationship of the cellular metabolic reactions of insect tissues to those already known for mammalian tissues. Furthermore, information obtained for insects and other lower forms is of value from the comparative standpoint and may provide the basis for an insight into the mechanism by which energy is provided for certain specialized behavior patterns in insects such as the cockroach.

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[2] Present address: Department of Biochemistry, University of Minnesota, St. Paul.

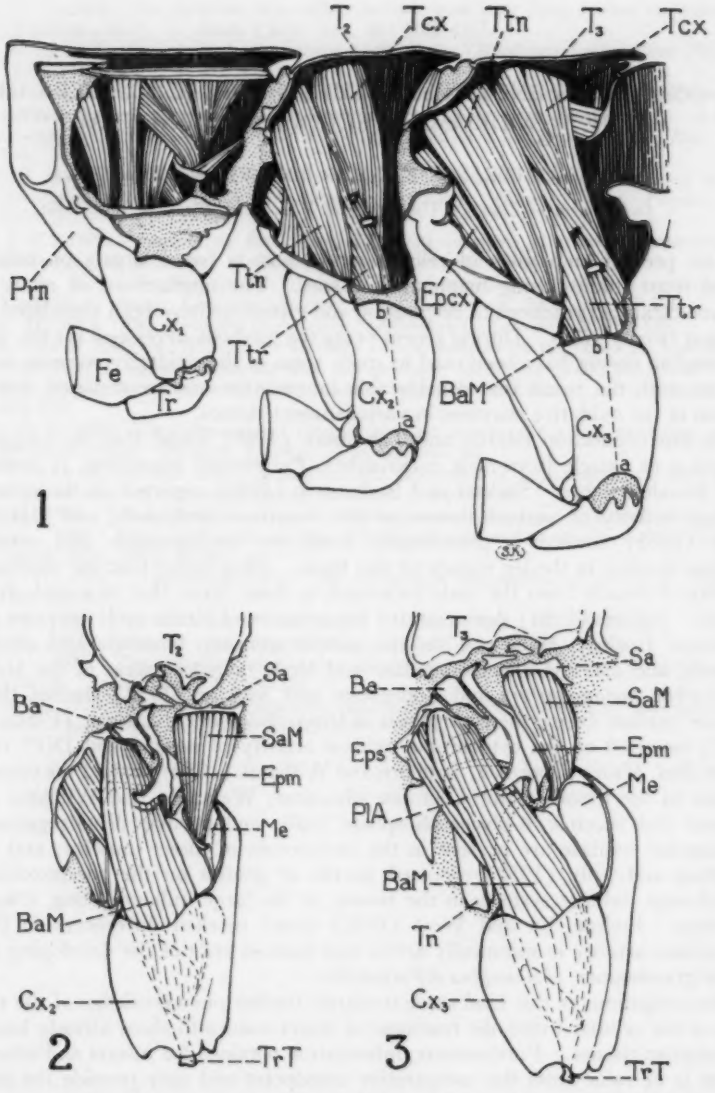

FIGURE 1. Mesal view of the right half of the thorax of the woodroach, *Leucophaea maderae*, showing some of the pigmented mesothoracic and metathoracic muscles (*BaM*, basalar muscle; *Tcx*, tergo-coxal muscle; *Ttn*, tergo-trochantinal muscle; *Ttr*, tergo-trochanteral apodeme muscle) used in the preparation of "leg muscle" homogenate. *a-b*, line of incision along coxae made to expose the entire muscles prior to removal; *Cx*, coxa; *Epcx*, episternalcoxal

The present paper reports results of a study of the succinoxidase, cytochrome oxidase and fatty acid oxidase of thoracic pigmented muscle from the woodroach, *Leucophaea maderae*. The mechanism of action and optimum conditions for these systems have been studied extensively in some mammalian tissues by Keilin and Hartree (1949), Slater (1949a, 1949b), Chance (1952), Lehninger (1946), and Lehninger and Kennedy (1948).

Materials and Methods

The woodroaches used in this study were isolated soon after metamorphosis and kept in dated containers so that muscle tissue could be obtained from roaches of known age. In certain of the earlier experiments, however, adult roaches of unknown age were used. Males and females were kept separately. All roaches were fed on the same constant dog pellet diet and ample food and water were always available to them.

The muscle tissue used was dissected from the meso- and metathoracic segments immediately after the roaches were killed by severing the head and abdomen. A mid-ventral incision through the thorax divided it into two halves. Remnants of the gut, large tracheal tubes and fat body were quickly cleaned away, and the large bundles of thoracic muscles were exposed as shown in Figure 1. Incisions along the meso- and metathoracic coxae along dotted lines *a-b* made it possible to separate these muscles in bulk with a few ventral and dorsal incisions. The tissue was weighed and placed in ground glass homogenizing tubes contained in an ice bath, and homogenized within 8 minutes after the roaches were killed. Sufficient water was added to give a 2.5 per cent homogenate which was used for the succinoxidase determinations. It was necessary to prepare a 0.5 per cent homogenate for the cytochrome oxidase and a 10 per cent homogenate for the determinations of fatty acid oxidase.

These muscles are sometimes referred to as the "leg muscles," and most of these muscles are in fact concerned with leg function. Roaches, such as the cockroach, *Periplaneta americana* as well as the woodroach, *Leucophaea maderae* and others, although comparatively weak flyers, can and do fly. Woodroaches in particular were observed on rare occasions to fly distances of 10–12 feet in slow, labored flight in the insectary. Further, Roth and Willis (1952) have shown that the wings of male *Periplaneta americana* and male *Blatta orientalis* are vibrated actively prior to copulation.

It is clear, then, that some muscles in roaches must function in flight and wing vibration. Carbonell (1947) in a detailed study of the thoracic musculature of the cockroach *Periplaneta americana* noted that these muscles bore little resemblance to

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muscle; *Fe*, femur; *Prn*, pronotum; T_2, mesothoracic tergum; T_3, metathoracic tergum; *Tr*, trochanter.

Figure 2. Mesal view of the mesothoracic flight muscles (*BaM*, basalar muscle; *SaM*, subalar muscle) which lie among the leg muscles and also included in the homogenate preparations. *Ba*, basalare; *Epm*, epimeron; *Me*, meron; *Sa*, subalare; *TrT*, trochanteral tendon. Other abbreviations as above.

Figure 3. Similar view of metathoracic flight muscles included in the homogenate preparations. *Eps*, episternum; *PlA*, pleural apodeme; *Tn*, trochatin. Other abbreviations as above.

those of other insects, and that in the musculature of the wings the cockroach thorax differs widely from the normal scheme of wing-bearing segments as given by Snodgrass (1935). The size of the basalar muscles (pronator-extensor of the wings) and the subalar muscles (depressor-extensor of the wings), which lie in the midst of the large leg muscles, led Carbonell to conclude that they must play an important role in flight.

Dissection of the woodroach revealed that prominent basalar muscles (BaM) and subalar muscles (SaM) are present in both the mesothoracic (Fig. 2) and metathoracic segments (Fig. 3). In fact, the basalar muscles[3] in both segments are the largest and longest of all the individual muscles present. These large flight muscles, together with the large leg muscles, are pigmented pink, in contrast to the smaller ventral longitudinal and certain smaller oblique muscles which are a translucent white color—and it was these pink pigmented muscles as a group which were used for the preparation of homogenates. Further, each homogenate represents not a mixture of muscles from several insects, but a preparation from the muscles of one roach of known age.

The enzyme determinations were made by use of the conventional Warburg apparatus. The homogenates were prepared by use of sharp-pointed, ground-glass homogenizers. The homogenates used for the study of succinoxidase and cytochrome oxidase were made with water and those for fatty acid oxidase with 0.154 *M* KCl. The proper amount of homogenate was placed in the flasks with the required cofactors for each of the enzyme systems studied. The flasks were placed in the bath at 38° C., and ten minutes were allowed for equilibration in the case of succinoxidase and cytochrome oxidase, and 6 minutes for fatty acid oxidase. Readings of oxygen consumption were taken at 10-minute intervals for at least 40 minutes. The average value for the number of 10-minute periods during which the oxygen consumption was constant, which was usually four periods, was used as a basis for calculating the Q_{O_2} values.

The methods used for the determination of succinoxidase and cytochrome oxidase were those reported by Schneider and Potter (1943). The optimum concentrations of required factors, and other conditions for maximum succinoxidase activity of thoracic muscle of the woodroach were determined. The concentrations of factors used for the cytochrome oxidase determinations were the same as those that have been reported for mammalian tissues. The fatty acid oxidase determinations were done by the method reported by Lehninger and Kennedy (1948). Final flask concentrations for the different enzyme systems are given in the footnotes to the tables.

The inhibitors were prepared in stock solutions which were in most cases 0.001 *M*. The solution of diethylstilbestrol was prepared by the procedure reported by McShan and Meyer (1946). The dry weight determinations of flight muscle were done by weighing the fresh tissue, placing it in a weighed tube and drying at 75° C. for 24 hours, after which the dry tissue was weighed and the weight used for calculating the percentage dry weight in terms of fresh weight.

Cytochrome *c* used for the determinations of succinoxidase and cytochrome oxidase was prepared by a modification of the method of Keilin and Hartree (1937),

[3] The basalar muscle actually arises from a tendon at the margin of the episternum adjacent to the basalare in each segment, but Crampton (1927) regards this margin of the episternum as an anterior portion of the basalare in the cockroach, *Periplaneta americana*.

or was obtained from the Sigma Chemical Company. Analytical reagent grade chemicals were used.

Results and Discussion

The succinoxidase activity of homogenates of thoracic muscle from female roaches of different ages was determined with different concentrations of succinate, phosphate buffer, calcium chloride, aluminum chloride and cytochrome *c*. The

Table I

Determination of the optimum concentrations of constituents required for maximum activity of succinoxidase in homogenates of woodroach thoracic muscle

Constituents			Concentrations of variable constituents and Q_{O_2} values**								
Absent	Present	Final *M* in flask									
$AlCl_3$	Phosphate pH 7.3	variable	*M**	0.0	0.0083	0.017	0.033	*0.050*	0.066	0.100	0.133
$CaCl_2$	Sod. succ. Cyto. *c*	0.1 2×10^{-5}	Q_{O_2}	37.0	40.0	69.0	83.0	*132.0*	115.0	115.0	98.0
$AlCl_3$	$CaCl_2$	variable	*M*	0.0	0.0004	0.0008	0.0012	*0.0016*	0.0020		
	Sod. succ. Cyto. *c* Phosphate	0.1 2×10^{-5} 0.05	Q_{O_2}	107.0	135.0	158.0	193.0	*208.0*	199.0		
$CaCl_2$	$AlCl_3$	variable	*M*	0.0	0.0004	0.0008	0.0012	*0.0016*	0.0020		
	Sod. succ. Cyto. *c* Phosphate	0.1 2×10^{-5} 0.05	Q_{O_2}	126.0	175.0	177.0	166.0	*186.0*	177.0		
None	Sod. succ.	variable	*M*	0.025	0.050	0.075	0.100	0.125	0.150	0.175	*0.200*
	Cyto. *c* Phosphate $CaCl_2+AlCl_3$ each	2×10^{-5} 0.05 1.6×10^{-3}	Q_{O_2}	99.0	125.0	142.0	160.0	174.0	189.0	199.0	*199.0*
None	Cyto. *c* ($\times 10^{-5}$ *M*)	variable	*M*	0.5	1.0	1.5	*2.0*	2.5	3.0		
	Sod. succ. Phosphate $CaCl_2+AlCl_3$ each	0.1 0.05 4×10^{-4}	Q_{O_2}	94.0	113.0	128.0	*131.0*	115.0	103.0		
None	pH	variable	pH	6.38	6.76	7.17	*7.3*	7.59	7.91		
	Sod. succ. Phosphate $CaCl_2+AlCl_3$ each Cyto. *c*	0.1 0.05 4×10^{-4} 2×10^{-5}	Q_{O_2}	94.0	112.0	128.0	*131.0*	115.0	103.0		

* Final molarity in flask.

** Q_{O_2} values are based on a dry weight content of 18.2 per cent and are averages of 2 to 5 runs using 0.1 ml. of 2.5 per cent homogenate except 0.2 ml. was used when cytochrome *c* and the pH were varied.

results italicized in Table I indicate the concentrations which gave maximum activity are 0.2 *M* succinate, 0.05 *M* phosphate buffer of pH 7.3, 1.6×10^{-3} *M* of each calcium and aluminum chlorides, and 2×10^{-5} *M* cytochrome *c*. Results of runs made at different pH values show that maximum activity was obtained at pH 7.3.

The data of Table II (Experiments 1 to 3) show that under the above conditions the oxygen consumption was directly proportional to the amount of tissue reacting

for 0.05, 0.10 and 0.15 ml. of 2.5 per cent homogenate but not for 0.20 and 0.25 ml. The results from Experiments 1, 2 and 3 are shown graphically in Figure 4. When 0.175 *M* succinate was used (Experiment 4, Table II) there was not quite a direct proportionality between the oxygen consumption and the amount of tissue reacting. These results indicate that a much higher concentration of succinate is required for optimum activity of the succinoxidase system of woodroach muscle than for this system of other tissues such as rat liver which requires only 0.05 *M* (Schneider and Potter, 1943). Harvey and Beck (1953) found 0.11 *M* succinate optimum for American cockroach muscle.

Essentially no oxygen was consumed when the succinoxidase system was run without substrate, and without tissue. When the cytochrome *c* was left out of the

TABLE II

Relation of oxygen consumption to amount of woodroach thoracic muscle used in the succinoxidase system

Amount of tissue* ml. 2.5% homogenate	Experiment No.							
	1(3)**		2(3)**		3(1)**		4(1)**	
	Oxygen consumption							
	Cmm. per 10 min.	Q_{O_2}	Cmm. per 10 min.	Q_{O_2}	Cmm. per 10 min.	Q_{O_2}	Cmm. per 10 min.	Q_{O_2}
0.05	7.6	201.2	6.1	160.8			8.0	210.9
0.10	15.0	197.3	12.1	160.0	19.5	257.2	15.5	204.0
0.15	22.4	196.9	18.4	161.3	28.6	252.0	21.9	192.0
0.20			23.4	154.1	34.6	228.0		
0.25					39.0	205.7		

* Flask concentrations of the constituents used in the system were sodium succinate 0.2 *M* except it was 0.175 *M* for experiment 4, phosphate buffer of pH 7.3 0.05 *M*, aluminum and calcium chlorides each 1.6×10^{-3} *M*, cytochrome *c* 2×10^{-5} *M*. The muscle used in these experiments was taken from adult females of unknown age.

** Number of runs with two flasks per each amount of tissue. The Q_{O_2} values are based on the average oxygen consumption for the first four 10-minute periods and a dry weight content of 18.2 per cent.

system the average oxygen consumption per 10 minutes was 2.9, 5.3 and 7.5 $mm.^3$, respectively, for 0.05, 0.10 and 0.15 ml. of 2.5 per cent homogenate. This was presumably due to the presence of cytochrome *c* in the flight muscle since this cytochrome has been shown to be present in cockroach muscle (Barron and Tahmisian, 1948; Harvey and Beck, 1953). Homogenates of woodroach muscle were therefore treated with sodium hydrosulfite to reduce the cytochromes and examined by use of a Hartridge Reversion Spectroscope. Absorption bands at the proper wave-lengths for cytochromes *a*, *b* and *c* were found, indicating the presence of these cytochromes in the woodroach muscle. On the basis of this evidence it appears that the activity of the system without added cytochrome *c* was caused by the presence of this component in the muscle homogenate.

The Q_{O_2} values of the different amounts of muscle homogenate used in Ex-

periments 1, 2 and 3 (Table II) were essentially constant and the average values were, respectively, 198.5, 161.0 and 254.6. The over-all average Q_{O_2} was 205. Adult female woodroaches of various ages were used for these experiments. These Q_{O_2} values are based on a dry weight of 18.2 per cent. The dry weights given in Table III, which range from 21.5 to 29.8 per cent, were done at different times than were those on which the above 18.2 per cent is based. The reason for this difference in the dry weights of these two series of experiments is not apparent.

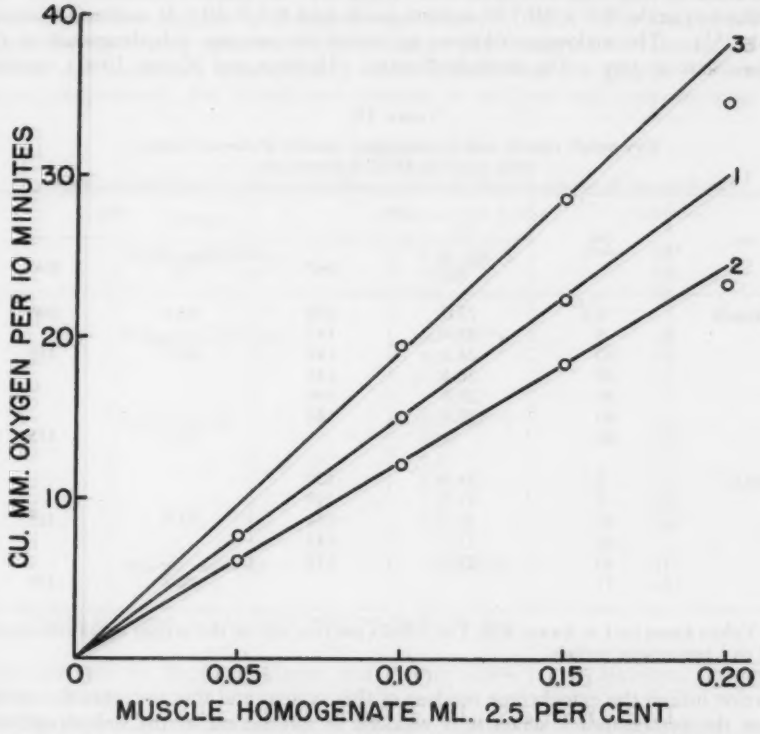

FIGURE 4. Results showing relation of different amounts of enzyme or tissue to oxygen consumption. Tissue concentrations which resulted in directly proportional oxygen uptakes are shown.

The roaches were kept under essentially constant conditions as to water, food and temperature, but factors other than these and the possible relation of dry weight to age may be involved.

The results given in Table III show the relation of age and sex to the dry weight and succinoxidase activity of woodroach muscle. There seems to be some tendency for the dry weight of muscle of both sexes to increase with increase in age after 5 days of adulthood. These results also show that there was an increase in

the succinoxidase activity of the muscle with increase in age of the roaches. Although most of these experiments were done during the spring of 1952, they were repeated for certain ages in the spring of 1953 with similar results, Table III). Further work, however, is necessary to clarify the variation in dry weight content of roach muscle.

The inhibition of the succinoxidase system of woodroach thoracic muscle was studied by use of diethylstilbestrol, cyanide, azide and malonate. Forty to 60 per cent inhibition was obtained with 0.27×10^{-4} *M* diethylstilbestrol, 0.5×10^{-4} *M* potassium cyanide, 0.5×10^{-3} *M* sodium azide and 0.5×10^{-2} *M* sodium malonate (Table IV). The malonate is known to inhibit the succinic dehydrogenase of the succinoxidase system. The diethylstilbestrol (McShan and Meyer, 1946), cyanide

TABLE III

Dry weight content and succinoxidase activity of thoracic muscle from woodroaches of different ages

Sex	Age days	1952		1953	
		Dry wt. %	Qo_2*	Dry wt. %	Qo_2*
Female	0.5	25.0	120	23.4	106
	5	23.9	143		
	10	24.2	148	24.5	110
	20	26.4	145		
	30	29.8	140		
	40	27.1	188		
	82			27.3	155
Male	0.5	24.3	129		
	5	21.5	149		
	10	23.7	154	23.9	128
	20	27.3	143		
	40	25.3	178		
	77			26.2	156

* Values based on 1 to 4 runs with 2 to 3 flasks per run, and on the oxygen uptake during the first 3 or 4 ten-minute periods.

and azide inhibit the cytochrome oxidase of this system and this prevents the oxidation of the cytochrome *c* when it is reduced by the action of the dehydrogenase. These inhibitors appear to affect the succinoxidase system of woodroach muscle in the same way as they affect this system in mammalian tissues.

Experiments were done to determine directly the succinic dehydrogenase activity of woodroach muscle by using brilliant cresyl blue (BCB) in the system as the mediator of hydrogen transport in place of the cytochrome system. When BCB was used in the system a Qo_2 of 76.5 was obtained as compared with 194 and 173, respectively, when cytochrome *c*, and BCB plus cytochrome *c* were present in the system. Similar results were obtained with rat liver which was run as a control. When cyanide was used in the system with BCB there was an increase of 83 per cent in the Qo_2 of woodroach muscle (Qo_2 of 76.5 for BCB alone) but under the same conditions cyanide did not cause an increase in the activity of rat liver. This in-

creased oxygen consumption when cyanide is added to the BCB system has been reported previously for leg muscle of the American cockroach (Harvey and Beck, 1953).

A Qo_2 of 1770 was obtained for the cytochrome oxidase of muscle from female roaches 30 days of age when 0.05 ml. and 0.1 ml. of 0.5 per cent homogenate were used per flask. Each flask also contained final concentrations of 0.033 *M* phosphate buffer of pH 7.3, 0.0114 *M* ascorbic acid, 4×10^{-5} *M* aluminum chloride, and 8.7×10^{-5} *M* cytochrome *c* which are essentially the amounts of these factors used for rat liver cytochrome oxidase by Schneider and Potter (1943). The Qo_2 of 1770 obtained for woodroach muscle is close to that of 1520 reported by Harvey and Beck (1953) for cockroach muscle but is much greater than the Qo_2 of 377 and 387 found, respectively, for cytochrome oxidase of rat liver and corpora lutea from

TABLE IV

Effect of inhibitors on the succinoxidase system of thoracic muscle of the woodroach

Inhibitor	Concentration in flask *M*	Inhibition %
Diethylstilbestrol	0.27 (10^{-4})	59*
	0.50	92
	1.0	96
Potassium cyanide	0.5 (10^{-3})	40
	1.0	93
	10.0	97
Sodium azide	0.27 (10^{-3})	44
	0.50	56
	1.0	61
	1.3	79
	2.7	80
	5.8	78
Sodium malonate	0.5 (10^{-2})	41
	1.0	60

* Values are based on 4 to 7 runs with two flasks per run.

pregnant rats by McShan, Meyer and Erway (1947) and McShan, Erway and Meyer (1948).

The fatty acid oxidase activity is low as compared to the succinoxidase activity of woodroach muscle, and there does not appear to be a significant change in activity with increase in age of the roach (Table V).

The results of this study show that succinoxidase and cytochrome oxidase systems are present in the thoracic pigmented muscle of the woodroach, *Leucophaea maderae*. In this muscle, however, the succinoxidase is more than twice as active and the cytochrome oxidase more than four times as active as in rat liver. On the other hand the fatty acid oxidase of rat liver is about ten times that of the woodroach muscle. Perhaps this is to be expected since the liver is known to be the locus for fatty acid metabolism.

Optimum conditions were determined for eliciting the maximum succinoxidase activity of woodroach muscle and it was found that this muscle requires four times

the concentration of succinate as does rat liver. In this connection Harvey and Beck (1953) found that the succinoxidase of leg muscle from the American cockroach requires 0.11 *M* succinate which is more than double that required by rat liver (Schneider and Potter, 1943). These results suggest that tissues high in succinoxidase, such as roach muscle, require higher concentrations of succinate for maximum activity than do tissues which contain a lower concentration of this system.

The results obtained with the BCB system, inhibitors and the required cofactors indicate that the mechanism of action of woodroach muscle succinoxidase is similar to that of mammalian tissues.

The increase in the succinoxidase activity of woodroach muscle with increase in age and the possible trend toward an increase in dry weight with increase in age may have physiological significance which is not apparent at present. In this connection Sacktor (1951b) showed that the cytochrome oxidase activity of normal and DDT-resistant house flies changes during pupal development, and Watanabe and Williams (1951) have reported differences in the cytochrome oxidase activity

TABLE V

Fatty acid oxidase activity of thoracic muscle from female woodroaches

Age in days	Q_{O_2}**
10*	3.1
20	5.8
40	3.0
60	4.5
Adult	3.7
Ave. for all ages	4.0

* When water was used for homogenizing muscle from a roach 10 days old a Q_{O_2} of 2.3 was obtained as compared to 3.1 for 0.154 *M* KCl.

** The amount of muscle tissue used per flask was 0.25 ml. of 10 per cent homogenate made with 0.154 *M* KCl. The final flask concentrations of reagents were 0.033 *M* KH_2PO_4-K_2HPO_4 of pH 7.4, 0.002 *M* potassium octonoate, 0.07 *M* KCl, 0.013 *M* $MgSO_4$, and 6.6×10^{-4} *M* KATP. The Q_{O_2} values are based on a dry weight content of 18.2 per cent.

of sarcosomes of Phormia isolated from insects of different ages. Further, Harvey and Beck (1953) found that succinoxidase is three times as active in the thoracic muscle of the male as in the female American cockroach, *Periplaneta americana*. These results for *Periplaneta* have been confirmed in our laboratory. It is therefore of interest that the succinoxidase activity in the thoracic muscle of the woodroach, *Leucophaea maderae*, is essentially the same in both sexes.

SUMMARY

1. The thoracic muscle of the woodroach, *Leucophaea maderae*, was shown to contain high concentrations of succinoxidase and cytochrome oxidase and a low concentration of fatty acid oxidase as compared to rat liver.

2. The conditions required for optimum activity of the succinoxidase system were determined and it was found that this system requires four times the concentration of succinate as does succinoxidase of rat liver.

3. Succinoxidase activity of thoracic pigmented muscle in the woodroach is essentially the same in both sexes, whereas in the American cockroach, *Periplaneta*

americana, the activity is three times as great in the muscle of the male as in that of the female. These latter results with *P. americana* (Harvey and Beck, 1953) have been confirmed in our laboratory.

4. Results were obtained which indicate that the succinoxidase activity of woodroach thoracic muscle increases with increase in the age of the roach.

5. The results of studies with cofactors, inhibitors and the brilliant cresyl blue system indicate that the mechanism of action of the succinoxidase of woodroach muscle is similar to that of mammalian tissues.

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TIDAL RHYTHMICITY OF RATE OF WATER PROPULSION IN MYTILUS, AND ITS MODIFIABILITY BY TRANSPLANTATION [1, 2]

KANDULA PAMPAPATHI RAO [3]

Department of Zoology, University of California, Los Angeles, California

While studying the rate of water propulsion in *Mytilus californianus* (Rao, 1953) it was observed that the behavior of the mussels was not the same at different periods of the day. A detailed study revealed that these differences were of the nature of a tidal rhythm, with periods of greater activity, corresponding to the times of high tide, alternating with those of lesser activity, corresponding to the times of low tide in the area from which the animals were collected.

Since the discovery of a persistent tidal rhythm in *Convoluta roscoffensis* (Bohn, 1903; Gamble and Keeble, 1903), similar rhythms have been described for a number of marine organisms from nearly all groups, and these have been reviewed by Calhoun (1944) and Brown, Fingerman, Sandeen and Webb (1953). Several molluscs have been described as exhibiting tidal rhythmicity in their activity. *Littorina rudis,* which is covered by water only during the semilunar high, high tides, becomes active at 15-day intervals when kept in the laboratory (Bohn, 1904). Brown, Bennett and Graves (1953) report a long-term tidal rhythm in *Venus.* Gompel (1937, 1938) reported the occurrence of a persisting tidal rhythm of oxygen consumption in *Patella, Mytilus, Pecten* and *Cytherea* while in *Haliotis tuberculata* it was not so marked. Brown, Bennett and Webb (1953) found the same in the crab *Uca.*

In the following studies an attempt was made to learn something of the nature of this rhythm in *Mytilus,* using as an index of activity the rate of water propulsion.

It is a pleasure to acknowledge my indebtedness to Professor Theodore H. Bullock for helping me in the procurement of the material; offering me all the laboratory facilities; for his enthusiastic encouragement during the course of this investigation and for critically reading through this paper. To the Chairman and Secretary of the Department of Zoology, and the other members of the staff, I am most grateful for several courtesies extended to me during my stay in the Department. My especial thanks are due to Professor G. E. MacGinitie, Director of the Kerckhoff Marine Laboratory, Corona del Mar, California, and to his staff for allowing me to make use of their laboratory pier for the experiment in transplantation of mussels. Finally I should like to place on record the promptness with

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[3] Present address: Department of Zoology, Andhra University, Waltair, India.

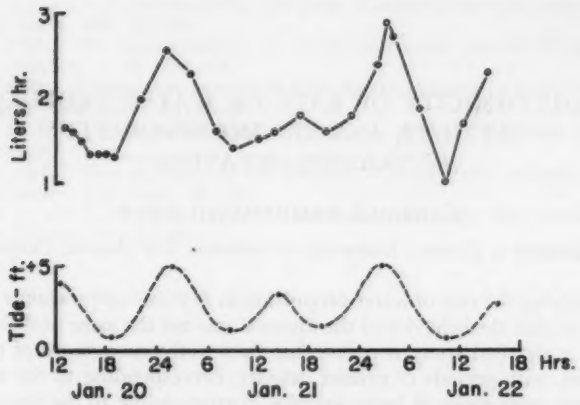

FIGURE 1. Variations in the rate of water propulsion in a single specimen of *M. californianus* collected inter-tidally from + 1.0 ft. and kept in darkness at 14 ± 1° C. Dotted line indicates the tidal cycle in the locality of collection, in this and the following figures.

which the Supply Department of the Marine Biological Laboratory, Woods Hole, Massachusetts, sent us the required supply of *Mytilus edulis*.

MATERIALS AND METHODS

Mytilus californianus collected from about + 1.0 ft. (tidal datum zero is mean lower low water, tidal range here about 8 feet) on pilings at Santa Monica, California, were transferred to aquaria containing sea water at 14 ± 1° C. One large mussel was placed in each of three enamel-coated pans containing sea water at 9 ± 1°, 14 ± 1°, and 20 ± 1° C., respectively, while a duplicate series of three pans contained ten to twelve mussels each, at the same three temperatures. All

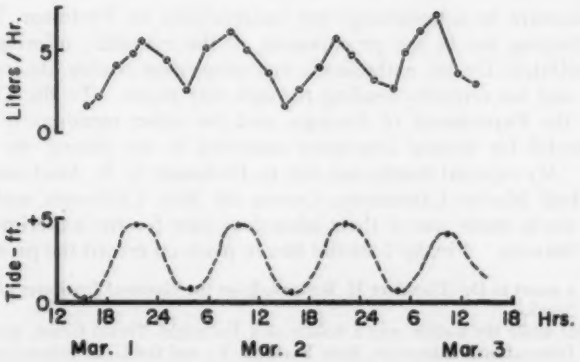

FIGURE 2. Rhythmicity in the rate of water propulsion in *M. californianus* collected from a depth of about 30 ft. off Los Angeles, and kept in darkness at 14° C.

the containers were covered with lids, making them virtually dark chambers. The method used for measuring the rate of water propulsion has been detailed elsewhere (Rao, 1953). Measurements were made at hourly intervals round the clock for 72 hours in continuity and this was repeated at three-day intervals, over a period of four to six weeks.

The same procedure as above was followed for *M. californianus* from about + 4.0 ft., on pilings and for *M. edulis* from pilings and from the underside of floats a few feet away. A collection of *M. californianus* obtained from a depth of about 30 ft. off the shore near Los Angeles, and a consignment of *M. edulis* collected at

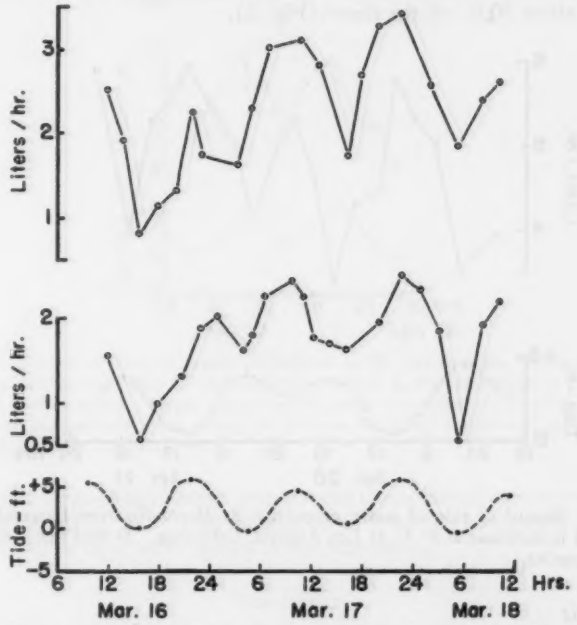

FIGURE 3. Variations in the rate of water propulsion over a period of 48 hours in *M. edulis* collected from floats and pilings at Santa Monica, California, and kept in darkness at 14° C. Upper graph for animals from floats and the lower one for those from pilings.

Barnstable Harbor on Cape Cod and flown to Los Angeles, California, were studied at 9 ± 1° C. and 14 ± 1° C.

Besides measurements on animals kept in continuous darkness, all the above samples were subjected to continuous light and the natural day and night environment and measurements made.

RESULTS

Mytilus californianus

Individuals of *M. californianus*, when observed in the laboratory, exhibit a pattern of activity (measured by the rate of water propulsion) which corresponds in

time and degree to the tidal levels in the locality from where they have been collected (Fig. 1). The pattern holds good even when several individuals are grouped together and their activity as a whole is measured. The rhythm is independent of temperature over the whole range measured, from 9 to 20° C. (as has been found by Brown, Bennett and Sandeen, 1953, in the fiddler crab) and persists for over four weeks in the laboratory in continuous darkness or continuous light, or the normal day and night environment. No indications of a diurnal rhythm in the rate of water propulsion were noticed.

Similar results were obtained regardless of the height inter-tidally from which animals were collected and even with mussels obtained from a sub-tidal population at a depth of about 30 ft. off the shore (Fig. 2).

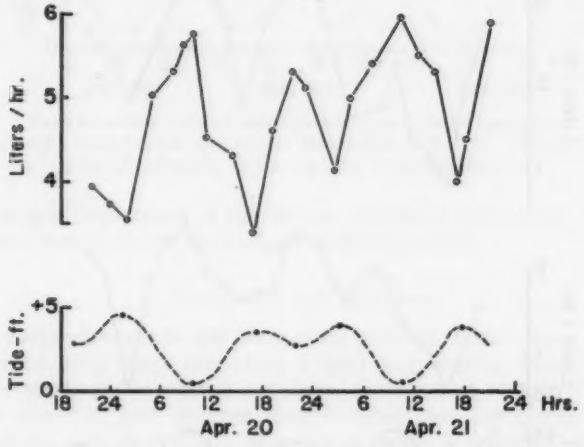

FIGURE 4. Record of rate of water propulsion in *M. edulis* from Barnstable Harbor on Cape Cod, kept in darkness at 9° C. at Los Angeles, California. Dotted line indicates the tidal cycle at Los Angeles.

Mytilus edulis

Samples of *M. edulis* collected from the same locality and treated similarly showed a tidal rhythmicity in their rate of water propulsion. What is more remarkable, mussels collected from the underside of floats showed a pattern of activity which was quite parallel to that exhibited by mussels collected from the pilings nearby (Fig. 3).

M. edulis collected at Barnstable Harbor on Cape Cod and studied at Los Angeles, California—nearly 3000 miles west—showed a rhythm in their rate of water propulsion which was out of phase with the local tidal cycle by about 6½ hrs. (Fig. 4), and this difference persisted for over four weeks in the laboratory.

Of the mussels obtained from Cape Cod, one dozen were kept in a small wire cage and, during low tide, were secured at + 1.0 ft. to a piling of the pier at the Kerckhoff Marine Laboratory, Corona del Mar, California, to study the effect of the local tidal schedule on these mussels. After a week's sojourn at this place,

they were brought back to the laboratory along with a sample of local *M. edulis* attached to the same piling at the same inter-tidal height, which served as controls for the experimental animals. Study of the activity pattern (at 9 and 14° C.) revealed a prompt shift in the rhythm to synchronize with the local tidal cycle and

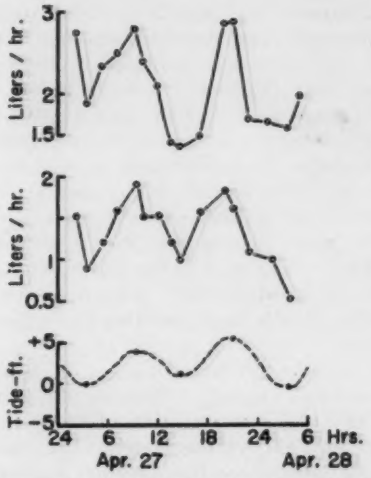

FIGURE 5. Record of rate of water propulsion in *M. edulis* from Barnstable Harbor on Cape Cod, after having been kept for one week at + 1.0 ft. in the inter-tidal at Corona del Mar, California, and of the control. Upper graph for *M. edulis* transplanted from Barnstable Harbor, and lower graph for mussels from pilings at Corona del Mar, California, serving as control. Dotted line indicates the local tidal cycle.

there is found to be good agreement between the transplanted east-coast mussels and the local controls (Fig. 5). They continued to keep in phase with the local tidal cycle for a period of over three weeks in the laboratory.

DISCUSSION

A marked tidal rhythmicity of rate of water propulsion is exhibited by populations of *Mytilus* occurring under a great variety of environmental conditions and persists in the laboratory for long periods (over four weeks) in phase with the tidal cycle of their natural environment, independent of a wide range of temperature (9 to 20° C.) and varying conditions of light and darkness. That it exhibits the same frequency in populations from high and low inter-tidal levels and even in sub-tidal populations (30 ft. deep) and that it persists in the laboratory, in phase with the tidal cycle outside, for long periods under constant conditions, demonstrate the intrinsic (or endogenous) nature of the rhythm.

It is most interesting that such a rhythm is evident in populations from the underside of floats (and hence not subject to the direct physical effects of the tides), with the same frequency and in phase with the local tidal cycle. It is equally of interest that a persistent rhythm with the same frequency, but out of phase with

the local tidal cycle, is exhibited by mussels removed nearly 3000 miles west from their natural environment. Such instances as these indicate that the rhythm, once set, is independent of external factors, such as cosmic influences, and can persist over long periods in the laboratory.

Instances like the foregoing demonstration of a tidal rhythm in a single species under a great variety of natural conditions lead one to suppose that organisms in general have rhythmic properties and that the frequency of the rhythm is intrinsic and perhaps inherited. But how such intrinsic rhythms at a given frequency come to be in synchrony with rhythmic events in nature is difficult to answer. But the ease with which they can be reset to suit a new environment, without a change in the frequency, though not abundantly demonstrated, is of sufficient significance inasmuch as it helps us to understand the existence of so many instances of tidal or other kinds of rhythmic behavior patterns. An intrinsic, inherited rhythmic pattern of activity is set in phase with external events of a rhythmic nature, which perhaps are of the same frequency as the organismic ones. Transplantation, as has been done for the first time in the above case, offers an ideal tool for studying this phenomenon in greater detail. Likewise, studying laboratory-grown individuals of species which show a rhythmic behavior in their natural environment, might yield fruitful results.

But the degree to which the rhythm is marked, perhaps, is dependent on the amplitude of the environmental rhythm. Thus the different findings (Bohn and Piéron, 1906; Bohn, 1906, 1907; Piéron, 1906, 1908; Gee, 1913; Parker, 1916; Crozier, 1921, and Hoffman, 1926) on the rhythmic behavior in sea anemones may be due to the fact that the intrinsic rhythm becomes marked and measurable only when the fluctuations of the environmental factors reach a certain, but unknown, threshold value.

Summary

1. The occurrence of a tidal rhythm in the rate of water propulsion in *Mytilus californianus,* collected from high and low inter-tidal levels and from a depth of 30 ft. off the shore, and also in *M. edulis* collected from pilings and the underside of floats, has been demonstrated.

2. Such a rhythm is independent of temperature (9 to 20° C.) and persists in the laboratory, in phase with the external tidal cycle, for over four weeks, in continuous darkness, or continuous light or the natural day and night environment.

3. No indications of a diurnal rhythm in the rate of water propulsion have been observed.

4. A rhythm of similar frequency, but out of phase with local tidal cycle by about 6½ hrs., was observed in *M. edulis* collected from Barnstable Harbor on Cape Cod and studied at Los Angeles, California, after transporting them by air.

5. Some of the east coast mussels were secured in the inter-tidal at Corona del Mar, California, for a week. Examination of their activity pattern after this period, revealed a prompt shift in their tidal rhythm to synchronize with the local tidal schedule.

6. The intrinsic nature of the rhythm is discussed and the probable inheritable nature of the rhythmic properties of organisms, coupled with the ease with which they could be set in synchrony with natural environmental rhythms, are suggested as likely causes for the widespread occurrence of rhythmic patterns in organisms.

7. It is suggested that the degree to which the intrinsic rhythm of the organism becomes marked and measurable depends upon the amplitude of the environmental rhythm.

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THE RESPIRATORY METABOLISM OF TISSUES OF MARINE TELEOSTS IN RELATION TO ACTIVITY AND BODY SIZE[1]

F. JOHN VERNBERG

Department of Zoology, Duke University, Durham, North Carolina, and Marine Biological Laboratory, Woods Hole, Massachusetts

Rates of oxygen uptake of tissues of fishes at different temperatures have been investigated by various workers (Fuhrman *et al.*, 1944, brain of large-mouthed bass; Peiss and Field, 1950, brain and liver of polar cod and golden orfe; and Freeman, 1950, brain and muscle of goldfish). In 1953 Vernberg and Gray reported a direct correlation between general body activity and oxygen metabolic rate of excised brain. They also noted that within the size range of animals used, no relationship between body size and rate of oxygen uptake was evident in the toadfish and the pinfish.

Although some workers reported a decrease in Q_{O_2} of tissues with increasing body size (Kayser, Le Breton and Schaeffer, 1925; Hawkins, 1928; Kleiber, 1941; Weymouth, Field and Kleiber, 1942; and Weymouth *et al.*, 1944), other investigators do not find this relationship to exist (Terroine and Roche, 1925; Grafe, 1925; Crandall and Smith, 1952; Bertalanffy and Pirozynski, 1953). Recently Krebs (1950), following a determination of the Q_{O_2} of five tissues of nine mammals, reported that there is not a simple correlation between body size and Q_{O_2} within the same species, and that, in general tissues of larger species have lower values than homologous values of tissues from smaller species.

The present investigation was undertaken for two specific reasons. First, to continue the study of the relationship of activity and metabolism of various tissues in marine fishes. Secondly, to examine the relationship of tissue metabolism and body size in a group of poikilothermic vertebrates.

Materials and Methods

The oxygen uptake of tissues was determined by the direct method of Warburg. Liver, muscle, and brain tissue from three species of marine teleost fishes, toadfish (*Opsanus tau*), scup (*Stenotomus chrysops*), and menhaden (*Brevoortia tyrannus*), were studied. These three species of fishes were used because of their diverse habits and differences in general activity levels. Menhaden is an extremely active swimming form which normally lives and feeds at the surface of the ocean. On the other hand, the toadfish is a relatively inactive pugnacious bottom-dweller, and the scup is intermediate to these two in respect to activity.

All animals were killed by severing the spinal cord in the region immediately posterior to the skull. Brain tissue was obtained by cutting off the roof of the skull and removing all tissue anterior to the vagal lobes. The brain was blotted

[1] Aided by a grant from the Duke University Research Council.

TABLE I

Respiration of tissues of three species of marine fishes

Species	N	Mean Q_{O_2}	Standard deviation
Brain			
Toadfish	28	6.78 ± .290	1.54
Scup	20	10.51 ± .578	2.59
Menhaden	21	13.04 ± .721	3.30
Liver			
Toadfish	27	4.42 ± .323	1.68
Menhaden	11	11.08 ±1.173	3.89
Scup	16	14.87 ±1.219	4.88
Muscle			
Scup	10	.410± .064	.202
Toadfish	18	.727± .084	.356
Menhaden	12	1.024± .140	.485

quickly on filter paper to remove all blood and foreign matter, then weighed and ground in a dry mortar. Sufficient amount of a phosphate buffer of pH 7.5 (glass electrode) was added to bring the volume to 3.0 ml. and the brei transferred to a Warburg flask. Muscles from the dorsal trunk region were treated in the same manner, using samples weighing about 450 mg. The liver tissue was sliced with a Stadie-Riggs tissue microtome; each sample weighed about 125 mg. The center well of the respirometer flask contained both 0.2 ml. of 10% KOH and filter paper wicks.

Time between the death of the animal and the beginning of the 10-minute period of thermal equilibration was kept constant at 10 minutes. Readings, taken at 10-minute intervals, carried for a minimum time of 60 minutes. Manometric determinations were made in a bath maintained at 30° C. Results are expressed in terms of wet weight Q_{O_2}. Thus Q_{O_2} denotes microliters of oxygen consumed per gram of wet weight per minute. The water content of the various tissues studied was determined by drying to a constant weight at 105° C.

This study was conducted at the Marine Biological Laboratory, Woods Hole, Mass., during the summer of 1953. All specimens were obtained from the Supply Department and maintained in the laboratory in aerated tanks supplied with running

TABLE II

Significance of differences of means of Q_{O_2} of tissue from marine teleost fishes

Tissue	Species compared	Probability	
Brain	Toadfish-scup	<.01	Highly significant
Brain	Menhaden-scup	<.01	Highly significant
Liver	Toadfish-menhaden	<.01	Highly significant
Liver	Menhaden-scup	.03	Significant
Muscle	Scup-toadfish	<.01	Highly significant
Muscle	Toadfish-menhaden	.07	Not significant

TABLE III

Water content of tissues of three species of marine fishes

Species	Tissue	No. of determinations	Average %	Range
Toadfish	Brain	7	83.19	81.1–84.7
	Liver	10	73.75	66.9–80.6
	Muscle	8	82.18	79.6–86.1
Scup	Brain	6	80.40	78.8–82.4
	Liver	8	76.40	71.4–78.8
	Muscle	8	78.58	75.4–80.6
Menhaden	Brain	10	78.94	75.3–81.4
	Liver	5	60.69	58.4–63.0
	Muscle	5	72.88	70.1–73.9

sea water. Scup and toadfish could be kept very well in these tanks but the menhaden would soon die of apparent oxygen lack. Thus it was necessary to use these animals as soon as they were brought into the laboratory. Menhaden, partially asphyxiated when brought from the traps, were not normal (Hall, Gray and Lepkovsky, 1926).

In the statistical analysis of the data pertaining to the relationship of Qo_2 to body size, the following formulae were used:

$$M = aW^b \tag{1}$$

or

$$\log M = \log a + b \log W, \tag{2}$$

where M is the Qo_2, W the body weight, and a and b are constants, indicating the intercept and the slope of the regression line in the log-log plot. Additional statistics calculated were the standard error ($S_{(\log y \cdot \log x)}$) and ρ (coefficient of correlation).

Weights of animals used are as follows: toadfish, average 349 gms., range 78–

TABLE IV

Statistical analysis of relation of Qo_2 to body size in tissues of two marine fishes

	N	a	b	$S(\log y \cdot \log x)$	ρ
Toadfish					
Brain	28	2.055	.202	.1115	.780
Liver	27	9.998	−.1448	.0972	.742
Muscle	18	.327	−.1182	.2800	.409
Scup					
Brain	20	4.795	.1504	.111	.568

586 gms.; scup, average 166 gms., range 83–462 gms.; and menhaden, average 345 gms., range 193–495 gms.

Results

The Q_{O_2} values of brain, liver and muscles are indicated in Table I. Significance of differences of means is shown in Table II.

In respect to brain tissue a definite correlation between animal activity and oxygen consumption is noted. This is in accord with previous reported results of Vernberg and Gray (1953). As shown in Table II, there is a significant difference between the mean Q_{O_2} of all three species.

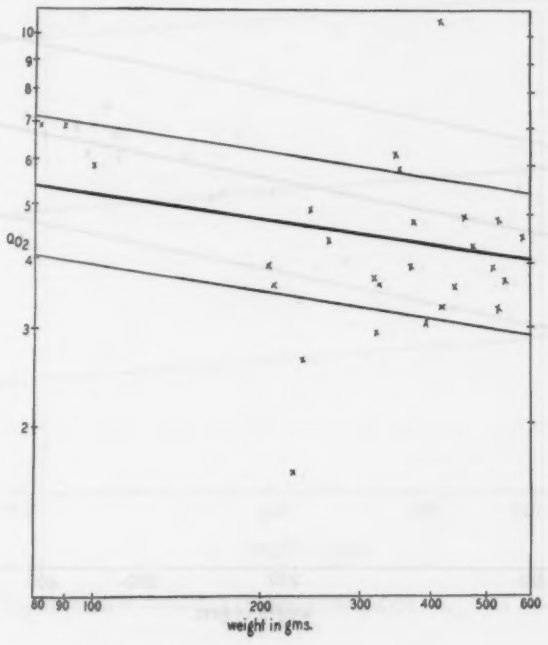

Fig. 1. Q_{O_2} of toadfish liver in relation to body size.

When comparing interspecifically the Q_{O_2} values of liver, no correlation between total animal activity and rate of oxygen uptake was noted. The liver of scup, the intermediate form in regard to activity, had a higher metabolic rate than liver of menhaden, the most active species. The degree of significance of difference of means is not as great when comparing menhaden and scup as when comparing menhaden and toadfish.

Scup muscle had the lowest Q_{O_2} values, menhaden the highest. However, there is no significant difference between means of toadfish and menhaden. As in

the case of liver, no correlation between animal activity and metabolic rate of muscle was noted.

Results of water content determination of the tissues studied are shown in Table III. In general intraspecific values were fairly constant; liver tissue showed the greatest variation. Interspecific comparison showed that similar values were obtained for brain and muscle tissues, but that the liver of menhaden had a much lower water content than either scup or toadfish livers. It is well-known that the liver of menhaden contains enormous quantities of oil and this probably accounts

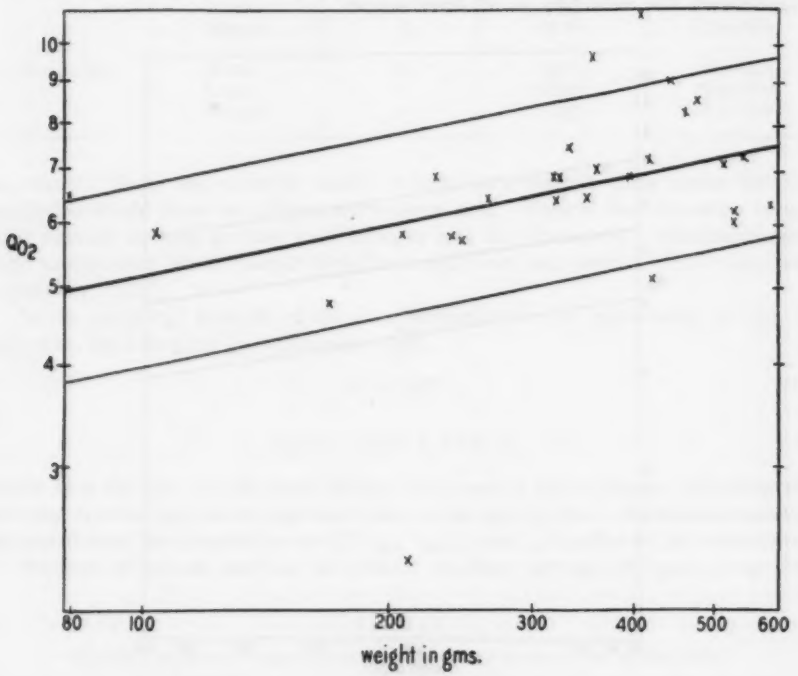

Fig 2. Q_{O_2} of toadfish brain in relation to body size.

for the lower water content. Only when comparing Qo_2 values of these three species would the significance between means be appreciably altered when results were based on dry weights. In this case the average Qo_2 values would be: toadfish 16.84 microliters/minute/gm. of dry weight, menhaden, 28.2, and scup 63.0. Thus, on this basis, the difference between scup and menhaden liver would be highly significant rather than significant.

Comparison of Qo_2 values for liver, brain and muscle from different individuals of the same species did not show any consistent tendency for one animal to have

a higher metabolic rate for all three tissues than another animal. The Q_{O_2} of brain of one animal may be higher and the liver Q_{O_2} lower than that of another.

The statistical analysis of the relation of Q_{O_2} to body size in toadfish and scup is presented in Table IV. Q_{O_2} values of menhaden were not evaluated because of the small size range of animals used (193–495 gms.). Figures 1–4 represent the log-log plot of Q_{O_2} values of various tissues against body weight. The middle line is the regression of Q_{O_2}, and the two outer parallel lines give the standard error in per cent, including ⅔ of the determinations.

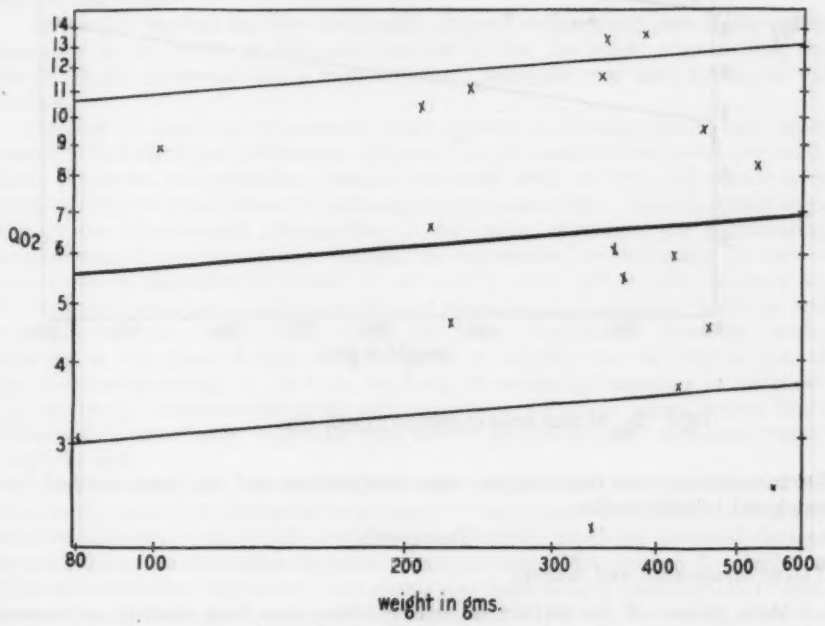

Fig. 3. Q_{O_2} of toadfish muscle in relation to body size.

Toadfish liver (Fig. 1). There is a slight decrease with increasing body weight but the correlation coefficient is low.

Toadfish brain (Fig. 2). A slight increase in Q_{O_2} values with increasing body weight is noted. In general these results correspond with the tendency observed by Bertalanffy and Pirozynski (1953) and Elliott (1948) for oxygen consumption of mammal brains.

Toadfish muscle (Fig. 3). Similar results to those of brain tissue.

Scup brain (Fig. 4). The same general tendency is noted for brain of scup as that of toadfish brain.

A significant difference in mean Q_{O_2} is noted between toadfish brain from Woods Hole and toadfish brain from Beaufort, N. C. (Vernberg and Gray, 1953).

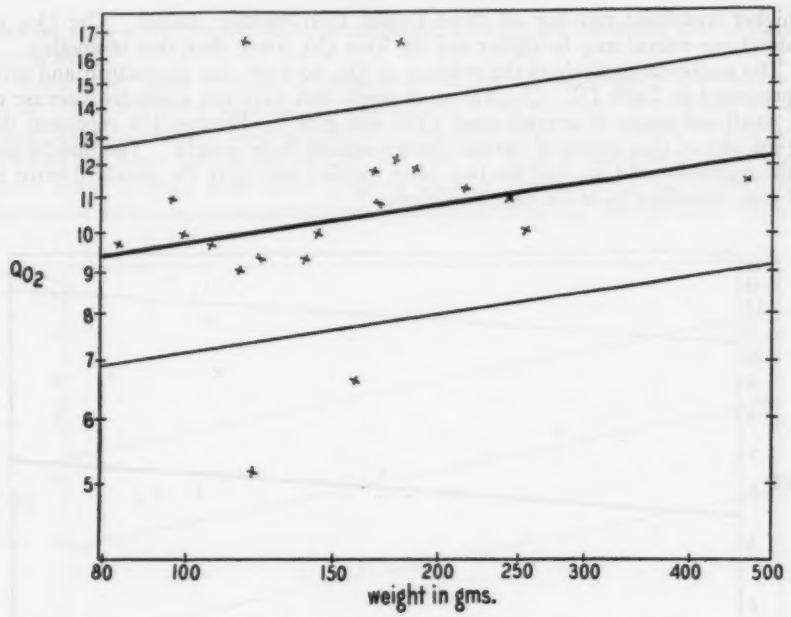

Fig. 4. Q_{O_2} of scup brain in relation to body size.

Determinations were made at the same temperature and the same method was employed in both studies.

Discussion

Tissue metabolism and activity

Many phases of the activity of marine fishes have been studied and certain physiological indexes have been correlated with their activity. A direct relationship between blood sugar concentration and activity was noted by Gray and Hall (1930); menhaden 75.2 mg.%, scup 52.6 mg.%, and toadfish 15.4 mg.%. Hall and Gray (1929) demonstrated a positive correlation between hemoglobin and activity: menhaden 41 mg. % iron, scup 25.3% iron, and toadfish 13.5% iron. A correlation between number of immature circulating erythrocytes and activity was shown by Dawson (1933): menhaden 16.5%, scup 4.7%, and toadfish less than 1%. Root (1931) studied the respiratory function of blood of marine fishes and found a definite adjustment on the part of the blood to the habits or characteristics of the fishes. His results, as they pertain to activity, are in agreement with those cited above.

Oxygen consumption determinations by Hall (1929) showed toadfish to have a low resting metabolic rate with a higher rate for scup. Menhaden have been found to have a high rate of oxygen consumption. In comparing gill area of

menhaden and toadfish, Gray (1947) found that the former has about 10 times more gill surface than the toadfish per gram of body weight and 15 times more gill area per square cm. of body surface. Gray (1946) found the scup to be intermediate to toadfish and menhaden in total number of gill lamellae. Thus, the physiological indexes of activity would substantiate field observations and indicate that menhaden is the most active form, toadfish the least active and scup intermediate. Vernberg and Gray (1953) found the brain of menhaden to have a higher Qo_2 than that of toadfish. The findings of the present paper demonstrate again the relationship of brain Qo_2 and activity for menhaden and toadfish and include results of another species, the scup.

In view of the fact that the comparative oxygen consumption rate of the entire organism for menhaden is high and toadfish is low, one might surmise that the tissues of the menhaden had a higher "basal" metabolic rate than tissues of the toadfish.

Because so much of an animal's body consists of muscle tissue, one might expect to find significant differences between Qo_2 of muscle of menhaden and toadfish. However, no correlation between metabolic rates of liver and muscle with either activity or total animal O_2 consumption was observed. Thus, it would seem that in the physiological organization of the entire organism, the coordinating mechanisms of the more active species, the menhaden, are operating in such a manner as to stimulate the tissues to an activity level higher than indicated by *in vitro* determinations. Many factors are operative in organismic make-up and would include such factors as hormonal and neural regulators. From the results reported in this paper it might seem possible to suggest that an integral part of the coordinating system of the body, the brain, is extremely important in maintaining the "basal" metabolic rate of the entire organism. Thus an animal having brain tissue with a high "basal" metabolic rate would have a high total organism "basal" metabolic rate.

Other workers have reported results which would indicate the importance of the brain to the general physiological functioning of the organism. In work with mammals by Himwich *et al.* (1939) and Hoagland (1949), rhythmic potential changes in brain tissue are dependent upon the metabolic rate of the tissue. A correlation of brain metabolism, respiratory movements and total oxygen consumption to temperature acclimatization was noted by Freeman (1950). He stated that the metabolic activity of the brain is a major factor in determining the level of the total oxygen consumption of a fish. The brain exerts this governing action through its influence on the other tissues of the body.

An interesting question remains to be investigated further. If the brain tissue Qo_2 is correlated with total oxygen consumption, why then should the Qo_2 of brain tissue be slightly increased in older animals, whereas, the Qo_2 of the whole animal is decreased. Undoubtedly the role of the other factors, such as endocrine relationships, must not be overlooked. Hoagland (1936) emphasized the modification of respiratory rhythms by reflexes and humoral agents.

Tissue metabolism and body size

The results of this study indicate that in a poikilothermic animal such as the toadfish, brain and muscle tissue Qo_2 values do not decrease with size as does liver,

but actually show a slight increase in "basal" metabolic rate with size. In general it would seem that any decrease in basal metabolic rate of the entire organism with increased size could not be accounted for on the basis of decline in muscle Qo_2. Bertanlanffy and Estwick (1953) reported that in the rat, although Qo_2 of muscle decreased slightly with body size, it was not of sufficient magnitude to account for decreased whole-animal oxygen consumption. Recently Bertanlanffy and Perozynski (1953) concluded, after investigating 7 different tissues of rats of various sizes, that any decline in basal metabolic rate depends not upon factors lying in the tissues themselves but rather on regulative factors in the organism as a whole. The present investigation would substantiate this view.

Geographical differences

Although the present study was not undertaken specifically to study geographic physiological adaptation, a significant difference in brain tissue metabolism of two populations of toadfishes was noted. The question arises as to whether this difference is due to genetic differences or to an acclimatization phenomenon.

Numerous workers have reported on the relationship of temperature acclimatization to whole animal oxygen consumption (Wells, 1935a, 1935b; Fry and Hart, 1948; Sumner and Doudoroff, 1938; Fox, 1936; and Fox and Wingfield, 1937). In general, animals from a northern habitat or acclimatized at lowered temperatures consume more oxygen when determined at intermediate or elevated temperatures than those that are from a southern area or acclimatized at a higher temperature. At the tissue level, Peiss and Field (1950) found that brain tissue from an arctic-adapted fish, the polar cod, had a higher metabolic rate than a warm-adapted southern species, the golden orfe, when determined at a temperature which corresponded to the acclimatization temperature of the warm- adapted animal. Freeman (1950), working with brain of goldfish, noted a similar relationship. The temperature of the water in which animals were kept averaged approximately 10° C. lower at Woods Hole than in the region of Beaufort. Thus, one would expect the brain Qo_2 of the northern population to be higher than the southern one. However, no attempt was made to study this phenomenon at different temperature levels or to investigate the possible genetic differences.

Summary

1. Determinations were made of the Qo_2 of brain, muscle and liver of three species of marine fishes representing different ecological habitats; a very active constantly swimming species, menhaden; a sluggish bottom-dweller, toadfish; and an intermediate form, scup.

2. Although a direct correlation between Qo_2 of brain and activity of the whole organism was noted, liver and muscle did not show any correlation with activity. The possible significance of this relationship was discussed.

3. A slight increase in Qo_2 of brain and muscle of toadfish and brain of scup with increasing body size was noted. The Qo_2 of toadfish liver decreased with body size.

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A PERSISTENT DIURNAL RHYTHM OF CHROMATOPHORIC RESPONSE IN EYESTALKLESS UCA PUGILATOR

H. MARGUERITE WEBB, MIRIAM F. BENNETT AND FRANK A. BROWN, JR.[1]

Department of Physiology and Bacteriology, Goucher College, Towson 4, Md.; Department of Biological Sciences, Northwestern University, Evanston, Ill.; and the Marine Biological Laboratory, Woods Hole, Mass.

On the basis of their response to eyestalk removal crustaceans have been classified into three types (Brown, 1948). The members of Group I, represented by *Palaemonetes,* respond to eyestalk removal by dispersion of the dark pigment and to injection of eyestalk extract by concentration of the pigment. *Crago,* the single member of Group II, responds to eyestalk removal by assuming an intermediate condition with the dark pigment partially dispersed. Group III contains all of the brachyurans except *Sesarma* (Enami, 1951) and is characterized by the complete concentration of the dark pigment on removal of eyestalks. Further investigations have led to the development of a concept of dual hormonal control in members of two of these groups. The evidence supporting such a concept for *Crago* has been reviewed by Brown (1948). In 1952, Brown, Webb and Sandeen demonstrated the presence in the central nervous system of *Palaemonetes* of a red-pigment-dispersing substance and adduced arguments in favor of its normal functioning.

The study of the mechanism of control of the melanophores of *Uca,* a representative of Group III, has been complicated by the presence in these animals of a persistent diurnal rhythm. Under the influence of the rhythmical mechanism the black pigment is dispersed by day and concentrated by night. The extent of dispersion is susceptible to modification by such factors as light, background, and temperature and in at least one species, *Uca pugnax,* an endogenous tidal rhythm has been shown to influence the condition of the chromatophores (Brown, Fingerman, Sandeen and Webb, 1953). All of these factors are thought to act on the chromatophores, at least in part, by virtue of alterations in the blood level of one or more hormones. The eyestalks are known to produce a hormone which causes dispersion of the black pigment. The central nervous system has been shown to contain a substance which disperses the black pigment of eyestalkless animals (Brown, 1948; Sandeen, 1950) but the participation of this substance in physiological color change has not been conclusively demonstrated.

Although all efforts at direct demonstration of a substance acting to concentrate the black pigment of *Uca* have ended in failure, there are cases in which investigators have been led to postulate the existence of such a substance (Brown and Stephens, 1951; Brown and Hines, 1952). Furthermore, Brown and Scudamore (1940) reported observations which suggested that eyestalkless *Uca* do not have their rhythm completely abolished.

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The data to be reported here contribute to our understanding of two problems: the mechanism of control of the black chromatophores of *Uca pugilator*, and the mechanisms involved in diurnal rhythmicity.

Experimental Procedure

All of these animals used in these experiments were specimens of *Uca pugilator* collected at Chapoquoit beach, near Woods Hole, Mass. during August, 1953. In the laboratory the animals were kept in white enamelled pans with a small amount of water and at a constant illumination of about 2 ft. c.

Two types of experiments were performed. One type involved a study of the changes occurring in chromatophores of legs which had been autotomized and were then maintained in sea water for a period of one hour. The other type of experiment consisted of injection of various concentrations of eyestalk extracts into eyestalkless animals.

Study of changes in chromatophores of isolated legs. Animals were forced to autotomize two or three legs each by applying pressure or by slightly injuring a distal segment of a walking leg. The legs so obtained were placed in sea water and observed at the time of isolation and again after thirty and sixty minutes. The total number of legs removed at any one time varied from six to ten and the legs were taken from two, three or five animals, depending upon the particular experiment. On each occasion a minimum of six legs from normal animals and the same number from eyestalkless animals were observed. Legs were isolated from two such groups at 66 different times; the total number of animals used was 226. The experiments were performed on four different days. In one series legs were removed every hour from 8 P.M. of one day until 8 P.M. of the succeeding day; in the other three series legs were isolated from both normal and eyestalkless animals as follows: 1) hourly from 8 A.M. till the next 1 A.M.; 2) hourly from 7 A.M. until the next 1 A.M.; 3) at 1, 2, 3, and 4 P.M. and at 8 and 9 P.M. of the same day. The eyestalkless donors had been operated on not more than 48 hours and not less than 8 hours before being used in an experiment.

Injection of eyestalk extract. A stock solution was made by grinding 10 dried eyestalks and extracting in one cc. of sea water. This solution was boiled for one minute and then cooled to room temperature. Five-hundredths cc. of this extract (the amount used for a single injection) contained ½ of an eyestalk or ¼ of the normal complement of eyestalk tissue of one animal. Such an extract is said to have a concentration of one quarter. This stock extract was then used to make up a series of concentrations as follows: 1/16, 1/64, 1/128, 1/512, 1/1024, and 1/2048. Seven groups of five eyestalkless animals were injected for each experiment. Each of the five animals in a group received 0.05 cc. of one dilution injected at the base of a walking leg. A control group of five animals received 0.05 cc. of sea water. The state of the chromatophores of each animal was determined at 15 minutes after injection, again at 30 minutes and at 30-minute intervals until they had returned to stage 1.

This experiment was performed four times, using two stock extracts. On one occasion a stock extract was made up in the afternoon, part of it was used immediately and the remainder refrigerated and used in the evening. Again an extract

was made up and part of it used in the evening while a second portion was refrigerated and used the next morning.

Since the chromatophores are initially in stage 1, the observed chromatophore stage minus 1 gives a measure of the dispersion present at any given time of observation. Summing the corrected values obtained during one experiment for any one concentration of extract then gives a measure of the activity of that extract. The activity of each extract tested was calculated in this manner.

Results and Discussion

Figure 1 shows the average stage of the chromatophores of legs isolated from eyestalkless animals, as determined 60 minutes after removal, plotted against time of day at which autotomy occurred. The data used for this curve are those obtained

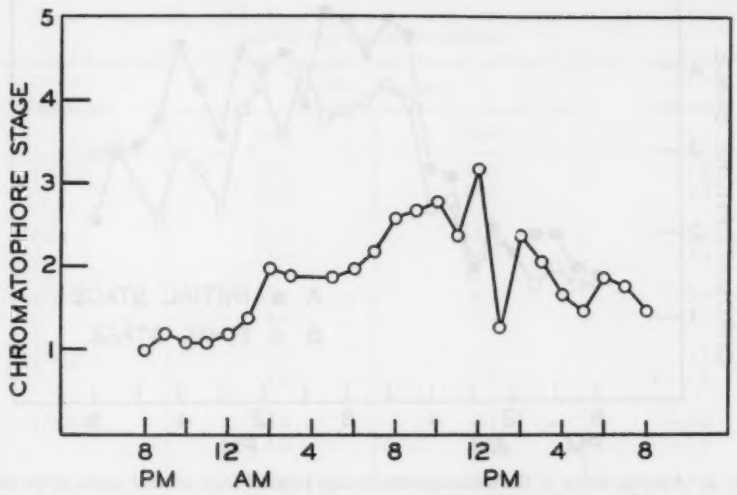

Figure 1. Average index of the melanophores in legs isolated from eyestalkless crabs sixty minutes after isolation at various times during a twenty-four hour period.

in the complete 24-hour series of observations. The other experiments of this type yielded entirely similar results. Since the initial average condition for all chromatophores in legs from eyestalkless animals was stage 1.0 the distance of any point from the abscissa gives a measure of the amount of dispersion occurring at that time of day. It can be seen that during the hours from 8 P.M. to 1 A.M. only very slight dispersion of the pigment occurs in 60 minutes. From 1 A.M. until 12 M. there is a gradual increase in the amount of dispersion observed, while from noon until 8 P.M. a gradually decreasing amount is found.

Curve A of Figure 2 represents the average initial stages of the chromatophores of legs isolated from intact animals plotted against the time of day of removal. Curve B of Figure 2 is obtained by similarly plotting the average stages, at 60

minutes after removal, of the chromatophores of legs from intact animals. These data were obtained on the same day and at the same times as those shown in Figure 1. Results obtained in other experiments of this series were similar to those represented in Figure 2. It can be seen that Curves A and B of Figure 2 are similar in general shape to that describing the conditions for eyestalkless animals. The values are low from 8 P.M. to 2 A.M., increase rather rapidly until about 5 A.M., remain fairly constant until 1 P.M. and then decrease gradually until 8 P.M. The distance between a point of Curve A and the point for the same hour on Curve B gives a measure of the change, concentration or dispersion, occurring in legs removed at that particular hour of the day. It is immediately obvious that the chromatophores in legs isolated from intact animals undergo more or less concentration throughout

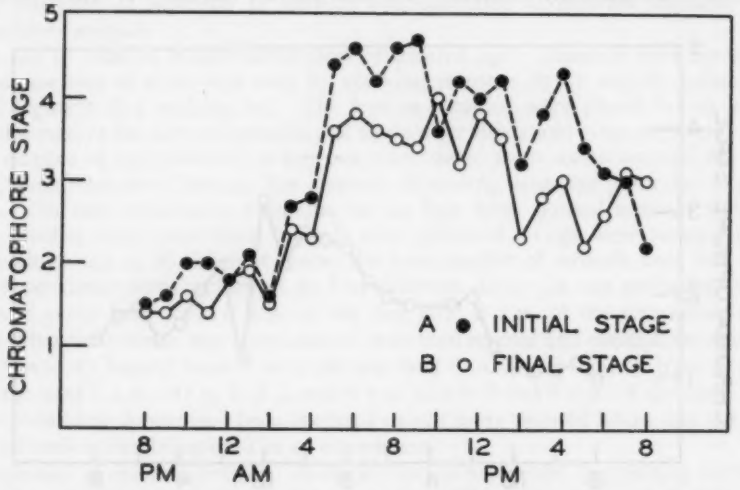

FIGURE 2. Average index of the melanophores in legs isolated from normal crabs at the time of isolation (A) and sixty minutes afterwards (B) as a function of the time of day.

most of the day. In only one case (10 A.M.) is there any noticeable degree of dispersion.

Since all of the isolated legs were kept in sea water and at a constant light intensity, any differences found among experimental groups must be accounted for in terms of differences in the body fluids at the time of isolation. The fact that the chromatophores of legs isolated from eyestalkless animals show different degrees of dispersion at the end of 60 minutes, depending upon time of day of isolation, clearly indicates a diurnal rhythm in eyestalkless animals marked by alterations in the body fluids of the animals.

The results of the injection experiments are presented in Table I. The activity values (calculated by the method previously described) are given for each of the four times the experiment was performed. It is seen that for each of the four lowest

concentrations the activity is lower at night than in the daytime. Although it is possible that some decrease in activity of the extracts occurred between the time of first preparing an extract and the time of injection any such decrease should tend to obscure the differences rather than accentuate them. Thus a decrease with time could result in relatively low daytime values when the extract was made up at night. When the extract was made up in the daytime and used at night the time elapsed was only six hours as compared with 12 hours when the reverse order was followed. The reduction in activity observed is such as would be expected by a two- to four-fold reduction in concentration.

The results obtained in both types of experiments clearly demonstrate that a diurnal rhythm exists in eyestalkless *Uca pugilator*. The changes observed in

TABLE I

Difference between day and night activity for a series of concentrations of eyestalk extract injected into eyestalkless animals

Concentration	Activity of extracts		Difference
	Day	Night	
1/16	18.8	20.0	1.2
	16.0	18.4	2.4
1/64	12.4	13.7	1.3
	12.0	9.0	−3.0
1/128	10.4	8.4	−2.0
	8.0	7.8	−0.2
1/512	6.0	2.2	−3.8
	4.8	3.6	−1.2
1/1024	1.8	0.2	−1.6
	2.6	0.8	−1.8
1/2048	1.6	0.0	−1.6
	1.0	0.0	−1.0

isolated legs show that the rhythm may be characterized as consisting of two distinct phases whose time relationships correspond quite closely with those of the rhythm found under the same conditions for normal animals. From the results obtained following injection of eyestalk extract it is not possible to describe the duration of the phases. The "night injections" were made in both cases shortly after 8 P.M. and it is clear that the response of the eyestalkless assay animals was different from that observed when injections were made in either morning or afternoon.

When an attempt is made to define the nature of the rhythm in terms of substances in the body fluid it is immediately obvious that two substances must be involved. A dispersing substance produced in the eyestalks has long been recognized and it is reasonable to assume that the disappearance of this substance

permits the concentration of pigment that occurs in legs isolated from normal animals. The absence, or presence in smaller concentrations, of dispersing hormone at night might be assumed but whether this is sufficient to account for the observations is questionable. If one postulates a single substance which causes dispersion of the pigment and which is present during the day and absent or reduced in amount at night then one is implicitly assuming that in the absence of any hormone the pigment will be concentrated. Following eyestalk removal the black pigment is maintained in the concentrated condition but disperses in isolated legs. If this concentrated condition is maintained by virtue of the absence of chromatophorotropic hormone then there is no logical explanation for the dispersion that follows isolation. The conclusion is therefore inescapable that the pigment is maintained in the concentrated condition by some factor which is present in the body fluid and which disappears gradually from the isolated legs. The central nervous system is a known source of dispersing hormone but at the present time no source of a black-pigment-concentrating substance has been demonstrated.

Assuming that there are two antagonistic substances which function in the control of the black chromatophores of *Uca pugilator* the rhythm of eyestalkless animals appears to consist of an increased amount of concentrating factor at night. The rhythm of normal animals appears to consist of the production primarily of dispersing hormone in the day phase and primarily of concentrating substance at night. Regardless of the site of production of the concentrating factor it seems likely that control of secretion is nervous and it is clear that the structures of the eyestalk are not essential for continued rhythmicity.

The results obtained on injection of eyestalk extract are consistent with the interpretation that eyestalkless animals possess in their body fluid at night a substance antagonistic to the dispersing hormone of the eyestalk. The fact that no difference was observed with the highest concentrations used may indicate that a maximum response was obtained even at night and that therefore no further response could be expected.

Summary

1. The responses of the black chromatophores of *Uca pugilator* as observed in legs autotomized and maintained in sea water are described.

2. The pigment in legs from normal animals in the day (dispersed) phase becomes concentrated after isolation; that from normal animals in the night (concentrated) phase remains concentrated.

3. The pigment in legs isolated from eyestalkless animals disperses in the daytime and fails to disperse when the legs are removed from 8 P.M. to 2 A.M.

4. The activity on eyestalkless animals of a series of concentrations of eyestalk extract was determined in the daytime and at night. Four of the six concentrations tested were found to be more effective in the daytime than at night.

5. The results clearly demonstrate the existence of a diurnal rhythm in eyestalkless animals and that the structures of the eyestalk are not necessary for this rhythm.

6. The data provide strong evidence that a black-pigment-concentrating substance participates in the regulation of the chromatophore system of these animals.

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